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(54) Title: MURINE MONOCLONAL ANTI-IDIOTYPE ANTIBODY 3H1

(57) Abstract

The present invention provides a monoclonal anti-idiotype antibody 3H1 that escapes immune tolerance and elicits a specific immune response to CEA in mice, rabbits, monkeys, and patients with advanced CEA-associated disease. This invention also provides compositions which can be used in the detection or treatment of CEA-associated tumors mimics a specific epitope on carcinoembryonic antigen and a hybridoma that produces 3H1.

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MURINE MONOCLONAL ANTI-IDIOTYPE ANTIBODY 3H1

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial No. 08/484, filed December
28, 1994, which is incorporated by reference herein in its entirety.

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STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY
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FIELD OF THE INVENTION

This invention relates to anti-idiotype antibodies. More specifically, it relates to
monoclonal anti-idiotype antibody 3H1 which elicits an immune response against a specific
epitope of carcinoembryonic antigen (CEA).

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BACKGROUND OF THE INVENTION

In spite of extensive medical research and numerous advances, cancer remains the second leading cause of death in the United States. Colorectal cancer is the third most common cancer and the second leading cause of cancer deaths. While the traditional modes

5   of therapy, such as surgery, radiotherapy and chemotherapy, are widely used and are in many instances successful, the still existing high death rate from cancers such as colorectal compels the need for alternative modes of therapy.

The immunotherapy of human cancer using tumor cells or tumor-derived vaccines has been disappointing for several reasons. It has been consistently difficult to obtain large

10   quantities of purified tumor-associated antigens which are often chemically ill-defined and difficult to purify. In addition, there remains the problem of the immunobiological response potential against tumor antigens, or in other words, the question of whether a cancer patient can mount effectively an immune response against his or her tumor. Tumor-associated antigens (TAA) are often a part of "self" and usually evoke a very poor immune response in a

15   tumor-bearing host due to tolerance to the antigens, such as T cell-mediated suppression. Immunobiologists have learned that a poor antigen (in terms of eliciting an immune response) can be turned into a strong antigen by changing the molecular environment. Changes of hapten carrier allow T cell helper cells to become active, making the overall immune response stronger. Thus, changing the carrier can also turn a tolerogenic antigen

20   into an effective antigen. McBridge et al. (1986) Br. J. Cancer 53:707. Often the immunological status of a cancer patient is suppressed such that the patient is only able to respond to certain T-dependent antigens and not to other antigen forms. From these considerations, it would make sense to introduce molecular changes into the tumor-associated antigens before using them as vaccines. Unfortunately, this is impossible to accomplish for

25   most tumor antigens, because they are not well defined and are very hard to purify.

The network hypothesis of Lindemann ((1973) Ann. Immunol. 124:171-184) and Jerne ((1974) Ann. Immunol. 125:373-389) offers an elegant approach to transform epitope

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structures into idiotypic determinants expressed on the surface of antibodies. According to the network concept, immunization with a given tumor-associated antigen will generate production of antibodies against this tumor-associated antigen, termed Ab1; this Ab1 is then used to generate a series of anti-idiotype antibodies against the Ab1, termed Ab2. Some of

5 these Ab2 molecules can effectively mimic the three-dimensional structure of the tumor-associated antigen identified by the Ab1. These particular anti-idiotypes called Ab2β fit into the paratopes of Ab1, and express the internal image of the tumor-associated antigen. The Ab2β can induce specific immune responses similar to those induced by the original tumor-associated antigen and can. therefore, be used as surrogate tumor-associated antigens.

10 Immunization with Ab2β can lead to the generation of anti-anti-idiotype antibodies (Ab3) that recognize the corresponding original tumor-associated antigen identified by Ab1. Because of this Ab1-like reactivity, the Ab3 is also called Ab1' to indicate that it might differ in its other idiotopes from Ab1. Anti-Id monoclonal antibodies structurally resembling tumor-associated antigens have been used as antigen substitute to induce anti-tumor

15 immunity in cancer patients. Herlyn et al. (1987) PNAS 84:8055-8059; Mittleman et al. (1992 PNAS 89:466-470; Chatterjee et al. (1993) Ann. N.Y. Acad. Sci. 690:376-278.

A potentially promising approach to cancer treatment is immunotherapy employing anti-idiotype antibodies. In this form of therapy, an antibody mimicking an epitope of a tumor-associated protein is administered in an effort to stimulate the patient's immune system

20 against the tumor. via the tumor-associated protein. WO 91/11465 describes methods of stimulating an immune response in a human against malignant cells or an infectious agent using primate anti-idiotype antibodies. However, not all anti-idiotype antibodies can be used in therapeutic regimens against tumors. Moreover, since different cancers have widely varying molecular and clinical characteristics, it has been suggested that anti-idiotype therapy

25 should be evaluated on a case by case basis, in terms of tumor origin and antigens they express.

Anti-Id monoclonal antibodies structurally resembling tumor-associated antigens have been used as antigen substitutes in cancer patients. Herlyn et al. (1987) PNAS 84:8055-8059; Mittleman et al. (1992) PNAS 89:466-470; Chatterjee et al. (1993) Ann. N.Y. Acad. Sci. 690:376-278. It has been proposed that the anti-Id provides a partial analog of the

5   tumor-associated antigen in an immunogenic context.

Carcinoembryonic antigen (CEA) is a 180,000-kiloDalton glycoprotein tumor-associated antigen present on endodermally-derived neoplasms of the gastrointestinal tract, such as colorectal and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is also found in the digestive organs of the human fetus. Circulating

10   CEA can be detected in the great majority of patients with CEA-positive tumors. Specific monoclonal antibodies have been raised against CEA and some have been radiolabeled for diagnostic and clinical studies. Hansen et al. (1993) Cancer 71:3478-3485; Karoki et al. (1992) Hybridoma 11:391-407; Goldenberg (1993) Am. J. Med. 94:297-312. As with most tumor-associated antigens which are seen as self-antigens by the immune system, cancer

15   patients are immunologically "tolerant" to CEA, possibly due to its oncofetal origin. Studies to date on patients with CEA-positive tumors have not demonstrated the ability to generate immunity to CEA. Thus, immunotherapy based on CEA has heretofore not been possible.

CEA nonetheless is an excellent tumor-associated antigen for active immunotherapy with anti-idiotype antibody. CEA is typically present at high levels on the tumor cell surface.

20   CEA is also one of the most well-characterized antigens, as its gene sequence is known and its three dimensional structures have been identified. CEA is a member of the immunoglobulin supergene family located on chromosome 19 which is thought to be involved in cell-cell interactions.

Inasmuch as some of the epitopes on CEA are shared by normal tissues,

25   immunization with intact CEA molecule might trigger potentially harmful autoimmune reactions. An immune reaction against a tumor-associated epitope, on the other hand, would be desirable. An appropriate anti-idiotype antibody would be an excellent candidate to

induce anti-tumor immunity in CEA positive cancer patients. A number of investigators

have generated anti-idiotype antibodies in rats, mice, baboons and humans that mimic CEA.

See, e.g., Hinoda et al. (1995) Tumor Biol. 16:48-55; Losman et al. (1994) Int. J. Cancer

56:580-584; Irvine et al. (1993) Cancer Immunol. Immunother. 36:281-292. However, given

5   the size of CEA (and likely numerous epitopes), and the fact that CEA is expressed on some

normal tissues, it was not known whether anti-idiotype antibodies would be effective in

eliciting an anti-CEA response that effects anti-tumor immunity.

Carcinomas of the gastrointestinal tract are often not curable by standard therapies.

Thus, new therapeutic approaches for this disease are needed. The present invention

10   overcomes the deficiencies in the prior art by providing a monoclonal anti-idiotype antibody

(3H1) as an antigen (Ag) substitute to induce anti-tumor immunity in gastrointestinal cancer

patients with advanced CEA-associated disease, such as colorectal cancer.

All publications cited herein are hereby incorporated by reference in their entirety.

15   SUMMARY OF THE INVENTION

The present invention provides a murine monoclonal anti-idiotype antibody, 3H1,

which is able to elicit an immune response against carcinoembryonic antigen (CEA). The

invention also provides the amino acid sequence of the variable regions of 3H1 and a

polynucleotide sequence encoding these variable regions of 3H1.

20   Accordingly, in one aspect, the invention includes a monoclonal anti-idiotype

antibody 3H1 produced by a hybridoma cell line ATCC No. HB12003 and progeny thereof.

In another aspect, the invention includes a hybridoma cell line designated ATCC No.

HB12003 and progeny thereof.

In another aspect, the invention includes a hybridoma cell line that produces a

25   monoclonal anti-idiotype antibody having a light chain variable region amino acid sequence

identical to SEQ ID NO:2 and a heavy chain variable region amino acid sequence identical to

SEQ ID NO:4.

In another aspect, the invention includes a monoclonal anti-idiotype antibody having a light chain variable region amino acid sequence identical to SEQ ID NO:2 and a heavy chain variable region amino acid sequence identical to SEQ ID NO:4.

Another aspect of the invention is an antibody having a light chain variable region encoded by a polynucleotide encoding an amino acid sequence identical to SEQ ID NO:2 and a heavy chain variable region encoded by a polynucleotide encoding an amino acid sequence identical to SEQ ID NO:4.

In another aspect, the invention includes pharmaceutical compositions and vaccines comprising an effective amount of monoclonal anti-idiotype antibody 3H1.

In another aspect, the invention also includes methods of eliciting an immune response in an individual with advanced CEA-associated disease. These methods entail administering to the individual an effective amount of 3H1.

In another aspect, the invention provides methods of detecting the presence of an anti-CEA antibody bound to a tumor cell comprising the steps of contacting the tumor cell with 3H1 for a sufficient time to allow binding to the anti-CEA antibody, and detecting the presence of any 3H1 which is bound to the anti-CEA antibody.

In another aspect, the invention provides a diagnostic kit for detection or quantitation of anti-CEA antibody. These kits contain 3H1 in suitable packaging.

Another aspect of the invention are methods of palliating CEA-associated disease in an individual having advanced CEA-associated disease. These methods entail administration of an effective amount of 3H1 to the individual.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the DNA sequence (SEQ. ID NO:1) and the amino acid sequence (SEQ. ID. NO:2) of the light chain variable region of 3H1.

Figure 2 depicts the DNA sequence (SEQ. ID. NO:3) and the amino acid sequence (SEQ. ID. NO:4) of the heavy chain variable region of 3H1.

Figure 3 depicts mouse and rat immunoglobulni kappa chain gene sequences, comparing the sequences within the kappa chain constant region for different strains and highlighting allotypic differences. Included are kappa chain constant region sequences for BALB/c (SEQ. ID NO:5), PL (SEQ. ID NO:6), SJL (SEQ. ID NO:7), and M. spretus (SEQ.

5   ID NO:8). The four genetic allotypes encode two protein allotypes. Other naturally occurring allotypes are possible. The figure is excerpted from Solin et al. (1993) Immunogenetics 37:401-407, which is hereby incorporated herein by reference.

Figure 4 depicts two allotypes of the mouse immunoglobulin heavy chain. The germ-line DNA sequence from newborn mice is shown (SEQ. ID NO:9), along with the encoded

10 protein (SEQ. ID NO:10). Shown in the line above is another protein sequence obtained from the mouse myeloma MOPC 21 (SEQ. ID NO:11). Other naturally occurring allotypes are possible. The figure is excerpted from Honjo et al. (1979) Cell 18:559-568, which is hereby incorporated herein by reference.

Figure 5 is a bar graph comparing the reactivity of 3H1 with various antibodies.

15 ^{125}I-3H1 was tested against a panel of mAb of various specificities belonging to major Ig subclasses by a direct binding RIA.

Figure 6 is a graph depicting inhibition of binding of radiolabeled 8019(Ab1) to semi-purified CEA by 3H1. Circles denote 3H1; squares denote 4EA2, and unrelated antibody. 3H1 inhibited the binding 100% beginning at a concentration of 25 ng.

20 Figure 7 is a graph depicting the inhibition of binding of 8019(Ab1) to 3H1 by CEA. Closed circles denote semipurified CEA; open circles denote a control glycoprotein that does not bind to 8019. Semipurified CEA at 2.5 µg inhibited the binding of anti-Id 3H1 to iodinated 8019 by 50%, whereas the unrelated glycoprotein even at higher concentration did not inhibit binding.

25 Figure 8 is a bar graph depicting binding of sera from mice immunized with 3H1 to CEA. First bar, PBS-BSA; second bar, anti-4EA2; third bar, pre-immune sera; fourth bar,

sera from mice immunized with 3H1. shows immunization with 3H1 induced antibodies that bound to insolubilized CEA.

Figures 9-1 to 9-4 depict FACS analysis of LS174-T cells reacted with 8019 (Ab1) (Fig. 5-1); sera from mice immunized with 3H1 (Fig. 5-2); pre-immune sera (Fig. 5-3) Sera

5   from 3H1-immunized mice showed distinct binding (5-2) that was similar to the binding pattern obtained with 8019 (Ab1) (5-1). No significant binding was obtained with human B cell lymphoma cells which do not express CEA (Fig. 5-4).

Figure 10 is a graph depicting inhibition of 8019 binding to LS174-T cells by sera from rabbits immunized with 3H1. Open circles denote 8019 (Ab1); closed circles denote

10 serum from rabbit #730; open squares denote serum from rabbit #729; open triangles denote pre-immune sera.

Figure 11 is a half-tone reproduction of an immunoblot showing binding of Ab3 in rabbit sera to CEA. All reactions were with semi-purified extract of CEA separated by SDS-PAGE. Lane 1. molecular weight markers; lane 2, CEA extract stained with Buffalo black;

15 lane 3, 8019; lane 4, rabbit sera (after immunization with 3H1); lane 5, pre-immune rabbit sera; lane 6, control sera from rabbits immunized with unrelated anti-Id 4EA2.

Figure 12 is a half-tone reproduction of an immunoblot showing binding of Ab3 in mouse sera to CEA. Lane 1, 8019 (Ab1); lane 2, monoclonal mouse Ab3; lane 3, control.

Figure 13 is a half-tone reproduction depicting an immunostained

20 (immunoperoxidase) normal and cancerous tissue sections with Ab3. The pattern of reactivity of Ab3 on both normal and malignant colonic tissues was almost identical to that obtained with Ab1.

Figure 14 is a half-tone reproduction depicting immunostained (immunoperoxidase) normal and cancerous tissue sections with Ab3. Reaction with 8019 (Ab1) resulted in the

25 staining of tumor cells as well as secreted mucinous materials whereas reaction with mAb Ab3 resulted in the staining of tumor cells with no staining of secreted mucin.

Figure 15 shows a schematic of the idiotype network for human gastrointestinal carcinoma.

Figure 16 is a graph depicting the inhibition of 3H1 binding to Ab1 (8019) by monkey (PRO 667) Ab3 sera by radioimmunoassay (RIA). Open squares denote
5   preimmunization serum; solid squares denote serum after the second injection; open circles denote serum after the third injection; open triangles denote serum after the fourth injection.

Figure 17 is a bar graph depicting binding of monkey Ab3 sera to purified CEA by ELISA. Open bars denote preimmune sera; diagonally hatched bars denote immune sera; horizontally hatched bars denote control sera. The solid circles connected by a solid line
10 denote unrelated antigen CEA.

Figure 18 is a graph depicting the kinetics of the binding of monkey Ab3 sera (PRO 667) to purified CEA by ELISA. Open squares with a center dot denote preimmune serum; open circles denote serum after the first injection; crosses denote serum after the second injection; open squares denote serum after the fourth injection.

15 Figures 19-1 and 19-2 are graphs depicting immune flow cytometry analysis of LS174-T cells with monkey Ab3 sera. In Figure 19-1, tumor cells were reacted with pre-immune sera and Ab3 sera (1:100 dilution) from monkeys immunized with 3H1. In Figure 19-2, MOLT-4 cells that do not express CEA were reacted with pre-immune and immune monkey Ab3 sera raised against 3H1.

20 Figures 20-1 through 20-4 are half-tone reproductions depicting immunoperoxidase staining of colonic adenocarcinoma and normal colon by monkey Ab3. Serial sections of tumor cells were stained with: 20-1, monkey Ab3 (50 µg/ml); 20-2, 8019 IgG$_1$ (50 µg/ml); 20-3, unrelated monkey Ab3 (50 µg/ml). Figure 20-4 shows staining of normal colon cells with monkey Ab3 (50 µg/ml).

25 Figure 21 is a half-tone reproduction of an autoradiogram of an SDS-PAGE gel with ^{125}I labeled CEA after immunoprecipitation with Ab3 monkey PRO 667 (lane 1), 8019 (lane 2), and Ab3 monkey treated with unrelated Ab2 (lane 3).

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Figure 22 is a graph depicting inhibition of Ab1 binding to LS174-T cells by purified monkey Ab3. Squares with a dot in the center denote purified Ab3 from monkey immunized with 3H1. Open circles denote purified Ab3 from monkey immunized with control 11D10.

Figure 23 is a graph depicting inhibition of Ab1 (8019) binding to 3H1 by human

5   Ab3 sera by RIA. Solid squares denote patient #1; solid triangles denote patient #2; open squares denote patient #3; open triangles denote patient #4; solid circles denote patient #5. The dotted line represents preimmune serum.

Figure 24 is a bar graph depicting the reactivity of human Ab3 with purified radiolabeled CEA. Each bar represents a sample, with samples 1-12 from patients 1-12,

10   sample 13 a PBS-BSA control, and sample 14 was Ab1 8019 (10 μg).

Figures 25-1 to 25-3 are reproductions of traces showing flow microfluorimetry analysis of reaction of CEA positive colon cancer cell line LS174-T with patients' Ab3 sera. Tumor cells were reacted with Ab3 sera from patients immunized with 3H1 (Fig. 25-1) and murine Ab1 (Fig. 25-2). In Figure 25-3), human B lymphoma cells that do not express CEA

15   (Raji) were reacted with Ab3. Solid line denotes Ab3 sera; dotted line denotes preimmune sera.

Figure 26 is a bar graph depicting inhibition of Ab1 binding to LS174-T cells by patients' Ab3. Solid bar denotes Ab3; hatched bar denotes Ab1.

Figure 27 is a half-tone reproduction of an autoradiogram of an SDS-PAGE gel

20   separating ^{125}I-labeled CEA after immunoprecipitation with 8019 (lane 1), Ab3 from patient number 1 (lane 2), Ab3 from patient number 2 (lane 3), and Ab3 from a patient treated with unrelated Ab2 (lane 4).

Figures 28-1 to 28-6 are half-tone reproductions depicting immunoperoxidase staining of autologous and allogeneic colonic adenocarcinomas and normal colon by Ab1 and

25   patients' Ab3. Serial sections were stained with: patients' Ab3 on autologous tumor (Fig. 29-1); patients' Ab3 on allogeneic tumor (Fig. 29-2); 8019 IgG$_1$ (Fig. 29-3); unrelated

patients' Ab3 on tumor sections as in Fig. 1 (Fig. 29-4); 8019 IgG$_1$ on normal colon

(Fig. 29-5); and patients' Ab3 on normal colon (Fig. 29-6).

Figures 29-1 and 29-2 are bar graphs depicting T-cell proliferation assays from two

patients (29-1, number 1; 29-2, number 12). For each figure, each pair of bars indicates the

5   extent of T-cell proliferation in the presence of: 3H1-Alugel (first pair); iso-allotype matched

control 4DC6-Alugel (second pair); purified CEA (third pair); purified bovine serum albumin

(BSA) (fourth pair); and phytohemagglutinin (fifth pair). For each pair, the dark (first) bar

denotes pre-immune sera; the hatched (second) bar denotes post-immune sera.

10   MODES FOR CARRYING OUT THE INVENTION

We have discovered a monoclonal anti-idiotype antibody, 3H1, which induces a

specific immune response against a distinct and specific epitope of carcinoembryonic antigen

(CEA), a tumor-associated antigen. This epitope is unique to CEA and is not present on

other CEA-related lower molecular weight members of this family which are also found on

15   normal tissues. The antigenic determinant as defined by the monoclonal antibody 8019

(Ab1) against which 3H1 was raised is absent on normal adult tissues as evidenced by

immunoperoxidase staining and hematopoietic analysis. A hybridoma that produces 3H1 has

been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive,

Rockville, MD, U.S.A. 20852 on December 15, 1995 under the provisions of the Budapest

20   Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes

of Patent Procedure. It was accorded Accession Number HB12003.

We have also found that 3H1 is effective in eliciting an immune response (humoral

and/or cellular) in individuals with advanced CEA-associated tumors. While not wishing to

be bound by a particular theory, one way that this may occur is that the 3H1 combining site

25   may present a region that partly resembles an epitope in CEA, in the context of other epitopes

which renders it more immunogenic. Thus, the antibody of this invention is useful for the

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treatment of CEA-associated tumors in these individuals. It is also useful for detection of Abl or Ab3.

As used herein, the terms "3H1", "3H1 antibody" and "3H1 monoclonal anti-idiotype antibody" are used interchangeably to refer to immunoglobulin produced by the 3H1

5   hybridoma cell line deposited with the ATCC. Also included in the definition of 3H1 are fragments produced by enzymatic cleavage and/or chemical treatment of intact antibody that comprise both the entire heavy and light chain variable regions of 3H1 and are capable of binding 8019 (Ab1) in a standard immunoassay, such as Fab, F(ab')$_2$, and F(ab').

In one embodiment, the invention includes a monoclonal anti-idiotype antibody

10 (referred to herein as an "anti-Id") produced by hybridoma cell line ATCC HB12003 or progeny thereof. Also included in this invention is a hybridoma cell line designated ATCC No. HB12003 and progeny thereof. As used herein, "progeny" of a hybridoma are descendants of a hybridoma, which may or may not be completely identical to the original (parent) cell due to mutation or other adaptation, but that produce a monoclonal antibody that

15 maintains the ability to escape immune tolerance, i.e., to cause an immune reaction against CEA.

Generation of Monoclonal Anti-Idiotype Hybridomas and Selection of 3H1

3H1 was obtained by using the 8019 antibody as immunogen for an anti-idiotype response. 8019 binds to a unique epitope of CEA that is not present on other members of the

20 CEA family, with virtually no cross-reactivity with normal adult tissues or hematopoietic cells including granulocytes. Koprowski et al. (1979) Somatic Cell Genet. 5:957; Mitchell (1980) Cancer Immunol. Immunother. 10:1.

Syngeneic BALB/c mice were immunized four times with 8019 (Ab1) and their spleen cells were fused with the non-secretory mouse myeloma P3-653 cells. The screening

25 procedure included four steps: (1) Positive selection for antibody binding to 8019 (2) Negative selection against antibody recognizing isotypic or allotypic determinants; (3) Positive selection for an ability to inhibit the binding of 8019 to CEA; (4) Positive selection

for an ability to induce a humoral immune response against the original tumor-associated antigen (CEA) in both mice and rabbits.

Several Ab2 hybridomas were obtained that were specific for the immunizing Id of 8019 and did not react with any isotypic or allotypic determinants. To determine whether the
5   anti-8019 were directed against the paratope of 8019, the binding of radiolabeled 8019 to plate bound CEA or the CEA-positive cell line LS174-T was studied in the presence of varying amounts of Ab2 hybridoma culture supernatants. With as little as 5 μl of culture supernatant, several of the anti-Ids tested inhibited the binding greater than 90%. Ab2 producing lines able to inhibit 8019 binding to CEA were grown and purified from ascites
10   fluid for further studies.

Different purified Ab2 were prepared as vaccines and injected into naive mice and rabbits. After 3 or more injections, serum samples were titered for the presence of Ab3 that bound not only to the immunizing Ab2, but also to CEA. The Ab2 reproducibly inducing the highest titer of Ab3 with the desired specificity was designated 3H1. Further details of the
15   method used to obtain 3H1 are provided in Example 1.

Ab3 produced in animals immunized with 3H1 has been further characterized. The immune sera from both mice and rabbits competed with Ab1 for binding to the CEA-associated cell line LS174–T and inhibited the binding of radioiodinated Ab1 to Ab2. This indicated that anti-anti-Id (Ab3) in mice and rabbits may share idiotopes with Ab1 (8019) and
20   they probably bind to the same epitope as Ab1.

Monoclonal Ab3 that bind to CEA positive antigen have also been obtained from mice immunized with 3H1. The Ab3 (both polyclonal and monoclonal) reacted with semi-purified CEA Ag by dot blot analysis and stained LS174–T cells by immunoperoxidase method. Administration of 3H1 to non-human primates (cynomolgus monkeys) also
25   generated an immune response (Example 3). Ab3 produced in response to 3H1 was specific for CEA.

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Importantly, although humans with CEA-associated tumors are tolerized to the CEA

antigen, we have discovered that 3H1 elicits an immune response in patients with advanced

CEA-associated disease. Twelve patients with CEA-positive advanced colorectal carcinoma,

and who had failed standard therapies, were administered 3H1. Nine of the twelve patients

5   developed antibodies that were anti-CEA (Figs. 23-27). In addition, seven of the twelve

patients displayed a cellular immune response as evidenced by a T cell proliferation assay

(Fig. 29). All seven of these patients had also developed an Ab3 response. This is the first

demonstration of breaking immune tolerance to CEA in patients with advanced disease. A

more detailed description of this study is found in Example 3.

10   We have also found the nucleic acid sequence encoding the light and heavy chain

variable regions of 3H1 and the amino acid sequence of the light and heavy variable regions

of 3H1. Thus, the present invention includes an anti-idiotype antibody having a light chain

variable region amino acid sequence identical to that depicted in Figure 1 (SEQ ID NO:2)

and a heavy chain variable region amino acid sequence identical to that depicted in Figure 2

15   (SEQ ID NO:4). The invention also encompasses an antibody having a light chain variable

region encoded by a polynucleotide sequence identical to that depicted in Figure 1 (SEQ ID

NO:1) and a heavy chain variable region encoded by a polynucleotide sequence identical to

that depicted in Figure 2 (SEQ ID NO:3).

An "identical" polynucleotide or amino acid sequence means that, when the

20   sequences are aligned, there is an exact match between bases (polynucleotide) or amino

acids.

Example 2 describes the cloning of 3H1 cDNA, from which the amino acid sequence

was deduced. To confirm our amino acid sequence, we sequenced the light and heavy chains

of 3H1 for 10-15 degradation cycles. The amino acid sequences obtained were identical to

25   that deduced from the cDNA sequence.

The invention also includes an anti-idiotype antibody having a light chain variable

region encoded by a polynucleotide encoding an amino acid sequence identical to that

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depicted in Figure 1 (SEQ ID NO:2) and a heavy chain variable region encoded by a polynucleotide encoding an amino acid sequence identical to that depicted in Figure 2 (SEQ ID NO:4). It is well within the skill of the art, given an amino acid sequence, to deduce a polynucleotide encoding the amino acid sequence.

5   Also included in this invention is a hybridoma cell line, samples of which are deposited in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD, U.S.A. 20852, and given the deposit number HB12003, and progeny thereof. The invention also includes a hybridoma cell line that produces a monoclonal anti-idiotype antibody having a light chain variable region amino acid sequence identical to that depicted

10   in Figure 1 (SEQ ID NO:2) and a heavy chain variable region amino acid sequence identical to that depicted in Figure 2 (SEQ ID NO:4). Plasmids encoding the light and heavy chain variable region amino acid sequences (along with a portion of the constant region) have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20852 on _____ under the provisions of the

15   Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. They were accorded Accession Nos. _____ and _____, respectively.

The invention also encompasses 3H1 conjugated to a label capable of producing a detectable signal. These conjugated antibodies are useful, for example, in detection systems

20   such as quantitation of Ab1 (and/or Ab3) or imaging. Such labels are known in the art and include, but are not limited to, radioisotopes, enzymes, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. The labels may be covalently linked to 3H1, or conjugated to the 3H1 through a secondary reagent, such as a second antibody, protein A, or a biotin-avidin complex. Methods of labeling antibodies are

25   known in the art and need not be described in detail herein.

Preparation of 3H1

The antibody of this invention can be obtained several ways. 3H1 can be produced from the hybridoma ATCC No. HB12003 described herein. Methods of antibody isolation are well known in the art. See, for example, Harlow and Lane (1988) Antibodies: A

5   Laboratory Manual, Cold Spring Harbor Laboratory, New York, and Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory. The antibody can be obtained from the hybridoma via tissue culture or from mouse ascites. These techniques are known the art. For example, the cells can be cultured in a suitable medium, and spent medium can be used as an antibody source. Optionally, matrix-coated channels or

10   beads and cell co-cultures may be included to enhance growth of antibody-producing cells. For the production of large amounts of antibody, it is generally more convenient to obtain an ascites fluid. Such methods are known in the art, and generally comprise injecting hybridoma cells into an immunologically naive histocompatible or immunotolerant mammal, especially a mouse. The mammal is optionally primed for ascites production by prior

15   administration of a suitable composition; for example, Pristane. Preferably, 3H1 is purified from BALB/c ascites using recombinant protein G-agarose chromatography followed by Protein-A-CL-sepharose 4B chromatography.

Alternatively, 3H1 can be chemically synthesized using techniques known in the art, for example, using a commercially available automated peptide synthesizer such as those

20   manufactured by Applied Biosystems, Inc. (Foster City, CA).

3H1 can also be obtained by employing routine recombinant methods such as described in Sambrook et al. (1989). For instance, a polynucleotide encoding either the 3H1 heavy or light chain can be cloned into a suitable expression vector (which contains control sequences for transcription, such as a promoter). The expression vector is in turn introduced

25   into a host cell. The host cell is grown under suitable conditions such that the polynucleotide is transcribed and translated into a protein. Heavy and light chains of 3H1 may be produced separately, and then combined by disulfide bond rearrangement. Alternatively, vectors with

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separate polynucleotides encoding each chain of 3H1, or a vector with a single

polynucleotide encoding both chains as separate transcripts, may be transfected into a single

host cell which may then produce and assemble the entire molecule. Preferrably, the host

cell is a higher eucariotic cell that can provide the normal carbohydrate complement of the

5   molecule. The 3H1 thus produced in the host cell can be purified using standard techniques

in the art. A polynucleotide encoding 3H1 for use in the production of 3H1 by any of these

methods can in turn be obtained from the hybridoma producing 3H1, or be produced

synthetically or recombinantly from the DNA sequence provided herein.

The 3H1 antibody is of the IgG1 mouse subclass, and may be isolated by any

10   technique suitable for immunoglobulins of this isotype. Purification methods may include

salt precipitation (for example, with ammonium sulfate), ion exchange chromatography (for

example, on a cationic or anionic exchange column run at neutral pH and eluted with step

gradients of increasing ionic strength), gel filtration chromatography (including gel filtration

HPLC), and chromatography on affinity resins such as protein A, protein G, hydroxyapatite,

15   and anti-immunoglobulin. 3H1 may also be purified on affinity columns comprising the

8019 paratope; for example, in the form of a purified Ab1 or Ab3.

If 3H1 is to be administered to an individual, 3H1 is preferably at least 80% pure,

more preferably at least 90% pure, even more preferably at least 95% pure as well as free of

pyrogens and other contaminants. In this context, the percent purity is calculated as a weight

20   percent of the total protein content of the preparation.

Uses for and Methods Using 3H1

3H1 has several uses. It can be used to elicit an immune response in an individual

having advanced CEA-associated disease and thus treat those individuals for CEA-associated

disease. Preferably, the immune response is anti-CEA. Further, 3H1 can be used to detect

25   antibodies that bind to CEA or 3H1. 3H1 may also be used to remove unwanted excess

labeled Ab1 from the circulation of patients previously treated with labeled monoclonal anti-

CEA antibodies. The label may be any label attached to the antibody suitable for its intended

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use, including, for example. radioisotopes, toxic moieties such as toxins, and drugs. 3H1 is also useful for enhancing tumor detection in imaging.

Thus, the present invention includes methods of eliciting an immune response in a individual with advanced CEA-associated disease. such as CEA-associated tumors that entail

5   administering an effective amount of 3H1 to the individual. Preferably, the response is the production of anti-CEA antibody. As used herein, an "individual" is a vertebrate, preferably is a mammal, and more preferably human. Mammals include, but are not limited to, farm animals, sport animals and pets. A "CEA-associated tumor" is one that contains an CEA antigen, especially expressed on the surface of tumor cells. As used herein, "advanced"

10   CEA-associated tumors means that there is detectable metastasis, that is, detectable tumor masses at sites other than the primary site of the tumor. Masses are preferably detected by imaging techniques known in the art such as X-ray or CT scan. An "effective amount" is an amount sufficient to elicit an immune response, whether humoral and/or cellular. An effective amount can be administered in one or more administrations. Preferably, the

15   immune response is the production of anti-CEA.

Suitable subjects for administration of 3H1 antibody may be identified by a number of different criteria. Experimental animals may be administered 3H1, for example, to study the effect of 3H1 on the immune response, or to obtain useful reagents, such as anti-CEA specific antibodies and cell lines.

20   In a preferred embodiment, 3H1 may be used for treatment of advanced CEA-associated disease, such as CEA-associated tumors. For treatment, an effective amount of 3H1 is administered to an individual with advanced CEA-associated tumor(s). As used herein, an "effective amount" for treatment is an amount sufficient to palliate the disease state. An effective amount can be given in one or more than one administration. Treatment

25   of individuals with advanced CEA-associated disease with an effective amount of 3H1 may have any of the following effects in comparison with other individuals who are not so treated: decrease the rate of progression of the disease, stabilize the state of disease, prevent spread

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and/or cause remission. As used herein. "advanced" CEA-associated tumors means that there is detectable metastasis, that is. detectable tumor masses at sites other than the primary site of the tumor. Masses are preferably detected by imaging techniques known in the art such as X-ray or CT scan.

5   To elicit an immune response or treat an individual for an advanced CEA-associated tumor, 3H1 is administered to an individual parenterally, preferably intracutaneously. Other routes of administration include. but are not limited to, intramuscular and intradermal. 3H1 can also be administered indirectly, by treatment of cultured cells followed by introduction of these cultured cells into an individual.

10   The amount of 3H1 administered depends upon several factors, such as the condition of the individual and the route of administration. Typically, the dose per administration will range from about 0.1 mg to about 20 mg. More preferably, the dose will range from about 0.5 mg to about 10 mg; more preferably, from about 1 mg to about 8 mg. Preferably, the dose is about 1 mg to about 4 mg. 3H1 is typically administered bi-weekly for four

15   injections, followed by monthly injections as required. Timing of subsequent injections (i.e., a maintenance dose) will depend. inter alia, upon the condition and response of the individual being treated. Ab3 levels can be monitored, preferably by the diagnostic methods described herein, to determine when maintenance (booster) administrations should be given, which could typically be about every three months.

20   Preferably, 3H1 is administered with a pharmaceutically acceptable excipient. A pharmaceutically acceptable excipient is a relatively inert substance that facilitates administration of a pharmacologically effective substance. For example, an excipient can give form or consistency to the vaccine composition, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for

25   varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Examples of pharmaceutically acceptable excipients are described in Remington's Pharmaceutical Sciences (Alfonso R. Gennaro, ed., 18th edition, 1990).

Preferably, 3H1 is used with an adjuvant which enhances presentation of 3H1 or otherwise enhances the immune response against 3H1. Suitable adjuvants include aluminum hydroxide (alum), QS-21 U.S. Pat. No. 5,057,540) DHEA (U.S. Pat. Nos. 5,407,684 and 5,077,284) and its derivatives (including salts) and precursors (e.g., DHEA-S), beta-2

5 microglobulin (WO 91/16924), muramyl dipeptides, muramyl tripeptides (U.S. Pat. No. 5,171,568) and monophosphoryl lipid A (U.S. Pat. No. 4,436,728; WO 92/16231) and its derivatives (e.g., Detox™) and BCG (U.S. Pat. No. 4,726,947). Other suitable adjuvants include, but are not limited to, aluminum salts, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium wall preparations, mycolic acid derivatives, nonionic

10 block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) Nature 344:873-875. For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant can be used. The choice of an adjuvant will depend in part on the stability of the vaccine in the presence of the

15 adjuvant, the route of administration, and the regulatory acceptability of the adjuvant, particularly when intended for human use. For instance, alum is approved by the United States Food and Drug Administration (FDA) for use as an adjuvant in humans. Preferably, alum-precipitated 3H1 is used. Preparation of aluminum hydroxide precipitated 3H1 is described in Example 3.

20   Alternatively, 3H1 can be encapsulated in liposomes. Liposomes suitable for packaging polypeptides for delivery to cells are known in the art.

3H1 can be heat treated before administration and the heat treatment can be in the presence of adjuvant, for example, alum. Heat treatment is preferably at 45° C for 30 minutes in a sterile vial in a water bath. For instance, 3H1 can be heated at about 40 to 80°C,

25 preferably 45°C to 60 °C, for a period of about 5 minutes to 2 hours, preferably 15 minutes to 1 hour. The heat treatment can occur anytime before administration. Preferably, heat

treatment is within 7 days of administration. Other heat treatment procedures can be used, as long as the desired activity of 3H1 is not significantly compromised.

For the purpose of raising an immune response, 3H1 may be administered in an unmodified form. It may sometimes be preferable to modify 3H1 to improve its

5   immunogenicity. As used herein, "immunogenicity" refers to a capability to elicit a specific antibody or cellular immune response, or both. Methods of improving immunogenicity include, inter alia, crosslinking with agents such as gluteraldehyde or bifunctional couplers, or attachment to a polyvalent platform molecule. Immunogenicity may also be improved by coupling to a protein carrier, particularly one that comprises T cell epitopes.

10   In order to determine the effect of administration with 3H1, the subject may be monitored for either an antibody (humoral) or cellular immune response against CEA, or a combination thereof.

To determine the level of CEA antibody (Ab3) in a biological sample, for example, serum or plasma is obtained from the subject. The sample may optionally be enriched for

15   immunoglobulin before the assay is conducted, although this is not usually required. If a mouse immunoglobulin (such as 3H1) is to be used as an assay reagent, the sample is preferably pretreated to remove anti-mouse immunoglobulin activity. This may be performed, for example, by depletion on a mouse immunoglobulin column, or by mixing non-specific mouse immunoglobulin into the sample and removing any immunoprecipitate

20   formed.

To conduct the assay, anti-CEA that may be in the sample is contacted with a non-limiting amount of an antigenic equivalent of CEA. This may be isolated CEA, nitrocellulose with CEA affixed by direct blotting or by transfer from a polyacrylamide gel, cells expressing CEA (such as LS174T cells), membrane preparations from such cells, or

25   fixed tissue sections containing CEA. Alternatively, an anti-idiotype, particularly 3H1, may be used.

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Once the immune complex has formed, it is generally separated from uncomplexed CEA analog, and the amount of complex present is determined. The complex may be separated, for example, by centrifugation to collect cells or an immunoprecipitate, or capture by a solid phase. The amount of complex present may be measured by providing the CEA

5   analog with a label either directly. or by incubating with a secondary reagent. Alternatively, a competition assay may be performed. in which the sample is first incubated with the CEA analog, and then a non-limiting amount of a labeled anti-CEA reagent is added which competes with the anti-CEA which may be present in the sample. Suitable labels include radiolabels, enzyme labels. fluorescent labels, and chemiluminescent labels. A standard

10   curve is constructed using solutions known to contain no anti-CEA, and solutions with various relative concentrations of anti-CEA, in place of the sample. The sample containing the unknown amount of anti-CEA is generally assayed in parallel, and the relative amount of anti-CEA contained therein is determined by comparison with the standard curve. Preferred assays for determining anti-CEA levels using 3H1 antibody are described in more detail in a

15   following section.

The isotype of the anti-CEA antibody may be determined by including in the immunoassay an isotype-specific reagents. either at the separation or the labeling stage. For example, anti-human IgG may be used to separate or detect antibody of the IgG class present in a clinical sample of human origin. Presence of specific anti-CEA of the IgG class

20   generally indicates a memory response. Presence of anti-CEA of the IgM class generally indicates ongoing immunostimulation, such as may be due to the presence of an CEA expressing tumor, or ongoing treatment with 3H1.

If desired, anti-CEA antibody detected in a biological sample may be further characterized; for example, by competition with anti-8019 (Ab1) to determine whether they

25   are specific for related epitopes on CEA. Competition assays between Ab1 and Ab3 are described in detail in the Example section.

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Anti-CEA antibody may also be tested to determine whether it is cytotoxic.
Complement mediated cytotoxicity (CMC) is determined. for example, by using CEA-
expressing target cells (such as LS174–T) labeled with ^{51}Cr. Labeling may be accomplished
by incubating about 10^6 cells with ~200 µCi Na$_2$^{51}CrO$_4$ for 60 minutes at 37°C, followed

5   by washing. The assay is conducted by incubating the antibody (or clinical sample
containing the antibody) with the target cells. The opsonized cells are then washed and
incubated with a source of complement; for example, guinea pig serum pre-adsorbed to
remove intrinsic antibody activity. After a suitable incubation period at 37°C, release of
^{51}Cr into the medium is determined and compared with that from unopsonized control cells.

10   Release of ^{51}Cr correlates with CMC activity.

Another way of characterizing the anti-CEA antibody is by testing its ability to
participate in an ADCC response (Cheresh et al. (1986), Cancer Res. 46:5112). Radiolabeled
CEA-expressing target cells are incubated with the anti-CEA (in the form of heat-inactivated
serum), and effector cells. Normal human peripheral blood mononuclear cells (PBMC) are

15   suitable effector cells, and preferably are used at an effector:target ratio of about 100. After
approximately 4 hours at 37°C, the proportion of released ^{51}Cr is determined as a measure of
ADCC activity.

The cellular immune response in a subject being administered 3H1 may be quantified
by conducting standard functional assays for specific T cell activity.

20   One type of assay measures T cell proliferation. In this test, peripheral blood
mononuclear cells (PBMC) are obtained from a whole blood sample collected from the
treated subject. For experimental animals, spleen cells may also be used. T cells may be
enriched, for example, by centrifugation on a gradient such as Ficoll™. The cells are then
cultured in the presence of CEA or (more usually) irradiated CEA expressing cells at various

25   concentrations. Preferably, the stimulator cells are autologous with the responder cells,
particularly in terms of histocompatibility Class II antigens.

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Another type of assay measures T cell cytotoxicity. In this test, an enriched T-cell population is used to effect lysis of ^{51}Cr-labeled CEA expression target cells, prepared as described above. Preferably, the effector cells are autologous with the target cells, particularly in terms of histocompatibility Class I antigens. The T cell population may

5   optionally be pre-stimulated with CEA or a relevant cell line. The T cells are then combined at various ratios with about 10^4 labeled target cells; for example, in wells of a microtiter plate. The plate is optionally centrifuged to initiate cell contact, and the cells are cultured together for 4-16 hours at 37°C. The percent specific release of ^{51}Cr into the medium is measured in comparison with labeled targets cultured alone (negative control) and targets

10 lysed with a detergent such as 0.1% Triton™ X-100 (positive control).

Other relevant measurements to determine the effect of 3H1 administration include clinical tests as may be appropriate in determining the progression of cancer of the suspected type. Such tests may include inflammatory indicators, mammography, and radioscintigraphy, such as are described elsewhere in this disclosure.

15 *Use of 3H1 to Conduct Immunoassays*

Another way that 3H1 can be used is to assay for the presence of an antibody or other immune component that binds to 3H1, or to CEA. Such components may be present following therapeutic administration of 3H1, or may spontaneously arise due to the presence of an CEA-expressing tumor in an immunocompetent host. Assays may be conducted on

20 biological samples, usually clinical samples. As used herein, the term "biological sample" includes cells in culture, cell lysates, cell supernatants, serum, plasma, biological fluids, and tissue samples.

In one embodiment of this invention, 3H1 is used to detect the presence of an anti-CEA, particularly anti-3H1 idiotype, that may be present in a biological sample. The sample

25 is suitably prepared before conducting the assay, optionally by enriching for antibody activity. If the biological sample is suspected of containing antibody activity against non-idiotypic regions of 3H1 (particularly anti-mouse immunoglobulin), it is preferable to remove

24

them or conduct the assay so as to avoid their detection. Anti-mouse immunoglobulin antibody can be removed from a sample, for example, by precipitation with normal mouse IgG or adsorption with a mouse Ig adsorbant. Binding of anti-mouse immunoglobulin antibody, particularly that specific for the Fc region, can be minimized by judicious choice of

5   the reagents of the assay. F(ab')$_2$ or Fab fragments of 3H1 and other mouse immunoglobulin reagents are especially appropriate.

After the sample is suitably prepared, it is mixed with a excess functional equivalent of 3H1 under conditions that permit formation of a complex between 3H1 and any anti-CEA that may be present. The amount of complex is then determined, and compared with

10   complexes formed with standard samples containing known amounts of anti-CEA in the range expected. Complex formation may be observed by immunoprecipitation or nephelometry, but it is generally more sensitive to employ a reagent labeled with such labels as radioisotopes like ^{125}I. enzymes like peroxidase and ß-galactosidase, or fluorochromes like fluorescein.

15   Antibody assays may be conducted in fluid phase. For example, anti-CEA may be mixed with labeled 3H1. Alternatively, the anti-CEA in the sample may be used to compete with a labeled anti-CEA for binding sites on 3H1. Generally, bound and unbound label is separated to quantitate the percent bound. Suitable separation methods include gel filtration chromatography, and precipitation with antibody against immunoglobulin of the species from

20   which the sample is obtained, optionally in the presence of polyethylene glycol. Alternatively, the proportion of bound and unbound label may be determined in situ, for example, using fluorescence/quench labeling pairs or enzyme/inhibitor labeling pairs. See, e.g., U.S. Patent 3,996,345 (Ullman et al.).

It is generally more convenient to conduct a capture assay using a reagent linked to a

25   solid phase, such as a polyethylene test tube, microtiter plate well, or magnetic bead. In a competition-type capture assay, unlabeled anti-CEA in the sample competes with a labeled anti-CEA reagent for binding to 3H1. The 3H1 may be attached directly to the solid support,

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or captured later. for example, using an anti-3H1. In this assay, the amount of label associated with the solid phase is inversely related to the amount of anti-CEA in the sample.

In the sandwich-type capture assay, anti-CEA is captured by 3H1 attached directly or through a secondary reagent to a solid phase. After washing, the anti-CEA is detected using

5   anti-immunoglobulin of the appropriate species, or a second 3H1 antibody, to which a label is directly or indirectly attached. Alternatively, the anti-immunoglobulin may be attached to the solid phase and labeled 3H1 is used to complete the sandwich. If the anti-immunoglobulin used is isotype-specific, then the class of the antibody may also be determined. In this type of assay, the amount of label associated with the solid phase

10   correlates positively with the amount of anti-CEA in the sample.

Other methods of measuring specific antibody are known in the art, and may be adapted to measure anti-CEA by using 3H1 as the target antigen. All such adapted methods are embodied in this invention. Further descriptions of particular embodiments are provided in the Example section.

15   3H1 may also be used to measure the level of cellular anti-CEA activity, particularly anti-3H1 idiotype. In a preferred example, 3H1 is used to identify anti-CEA T cells, defined for this purpose as lymphocytes expressing a T cell receptor that binds the 3H1 idiotype. 3H1 may be labeled and contacted with a population of cells suspected of containing anti-CEA T cells. Alternatively, unlabeled 3H1 may be mixed with the cells, and followed with a

20   labeled secondary reagent such as labeled anti-mouse immunoglobulin or protein A. Suitable labels for this purpose include radiolabels and fluorescent labels. The use of fluorescent labels would also allow anti-CEA cells to be separated from non-specific cells in a fluorescence-activated cell sorter.

Use of 3H1 to remove labeled Ab1

25   The invention also encompasses methods using 3H1 to remove a toxin and/or a label, for example radioactivity, from an individual who has received a labeled anti-CEA antibody (Ab1), for example, for radioscintiligraphy or radiotherapy. One problem common to use of

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antibody targeted radionuclides (i.e.. radioimmunotherapy) has been the presence of excess

Ab1 in the system which limits the dosage of radiolabeled antibody for treatment. Further,

effective imaging using radiolabeled antibodies is hampered due to excess circulating

radiolabeled antibody, which often takes several days to clear circulation and tissues. In

5   these methods of the present invention, 3H1 is administered to the individual at a specified

time after administration of the labeled anti-CEA. The intention is for the 3H1 to complex

with anti-CEA at sites other than the tumor, such as in the circulation and interstitial spaces,

and thereby promote its clearance. As a result, the level of labeled moiety (such as

radioisotope) in unaffected tissues is reduced, and the image of the tumor (in comparison to

10   neighboring tissues) is enhanced. Similarly, when radionuclides are given to subjects for

irradiation of a tumor site, it is desirable to reduce collateral exposure of unaffected tissue.

This invention thus includes methods of treatment in which a radiolabeled anti-CEA antibody

is administered in a therapeutic dose, and followed by a molar excess of 3H1.

In either of these applications, an amount of 3H1 is chosen that is in sufficient molar

15   excess over the labeled anti-CEA to locate and bind any anti-CEA that is not localized at the

tumor site. The timing of administration and amount of 3H1 will depend upon the nature of

the radiolabeled antibody, the type of radioisotope used and the condition of the individual.

Preferably, the molar ratio of 3H1 to the anti-CEA antibody is at least about 5:1, more

preferably about 25:1 to 200:1. Preferably, 3H1 is administered 5 to 24 hours after the

20   individual has received the anti-CEA antibody.

The invention also includes methods of detecting the presence of an anti-CEA

antibody bound to a tumor cell comprising the steps of treating the tumor cell with 3H1 for a

sufficient time to allow binding to the anti-CEA antibody, and detecting the presence of any

complex formed. The intention is for the 3H1 to detect anti-CEA that has pre-attached to the

25   tumor cell; or alternatively, to promote the binding of anti-CEA to the tumor cell by forming

a polyvalent anti-CEA/3H1 immune complex. In the former instance, the 3H1 is provided

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with a detectable label or a means by which a label can be attached. In the latter instance, either the anti-CEA or the 3H1 is provided with a label.

This strategy may be used, for example, to identify an CEA-bearing cell in an isolated cell suspension. The cells are incubated sequentially or simultaneously with anti-CEA and

5   3H1, washed, and then the labeled cells are detected. Preferred labels for this embodiment include fluorescent labels, such as fluorescein, rhodamine, and Texas red. Optionally, labeled cells may be separated from unlabeled cells; for example, by sorting in a fluorescence-activated cell sorter or by affinity separation, using any of the solid phase positive or negative immunoselection techniques known in the art.

10   The strategy may also be used, for example, to detect or image tumors in an affected subject. The anti-CEA and 3H1 are administered (usually sequentially) into the subject and allowed to accumulate at the tumor site. Suitable labels include radiolabels such as ^{111}In, ^{131}I and ^{99m}Tc. The tumor is then detected or visualized using standard techniques of radioscintigraphy.

15   ***Pharmaceutical compositions and vaccines comprising 3H1***

The present invention encompasses pharmaceutical compositions and vaccines containing 3H1. Such pharmaceutical compositions and vaccines are useful for eliciting an immune response, preferably an anti-CEA response, and/or treating an CEA-associated disease. These pharmaceutical compositions, comprised of an effective amount of 3H1 in a

20   pharmaceutically acceptable excipient, are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations or parenteral and nonparenteral drug delivery are known in the art and are set forth in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing (1990).

25   One type of pharmaceutical composition is a vaccine. Accordingly, the present invention also includes vaccines comprising an effective amount of 3H1. As used herein, a "vaccine" is a pharmaceutical composition for human or animal use, which is administered

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with the intention of conferring the recipient with a degree of specific immunological

reactivity against a particular target, or group of targets. The immunological reactivity may

be antibodies or cells (particularly B cells, plasma cells, T helper cells, or cytotoxic T

lymphocytes and their precursors or any combination thereof) that are immunologically

5   reactive against the target. For purposes of this invention, the target is tumor associated

antigen CEA or any related tumor antigen bound by 3H1. The immunological activity may

be desired for experimental purposes, for treatment of advanced CEA-associated disease, or

elimination of a particular substance. The vaccines can be used, inter alia, to elicit an

immune response in an individual with advanced CEA-associated disease. Preferably, the

10   immune response is the production of anti-CEA.

Preferably, a vaccine of this invention will include an adjuvant. Examples of

adjuvants have been discussed above.

The vaccines of the present invention are typically administered parenterally, by

injection for example, either subcutaneously, intramuscularly, intraperitoneally or

15   intradermally. Administration can also be intranasal, intrapulmonary (i.e.. by aerosol), oral

and intravenous. Additional formulations which are suitable for other modes of

administration include suppositories and, in some cases, oral formulations. The route of

administration will depend upon the condition of the individual and the desired clinical

effect.

20   Administrations can begin on a weekly or biweekly basis until a desired, measurable

parameter is detected, such as elicitation of an immune response (humoral and/or cellular).

Administration can then be continued on a less frequent basis, such as biweekly or monthly.

Preferably, the administrations are initially given biweekly for the first four administrations,

followed by monthly administrations.

25   The vaccines are administered in a manner compatible with the dosage formulation,

and in such amount as will be therapeutically effective. The quantity to be administered

depends on the individual to be treated, the capacity of the individual's immune system to

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synthesize antibodies, the route of administration, and the degree of protection desired.
Precise amounts of active ingredient required to be administered may depend on the
judgment of the practitioner in charge of treatment and may be peculiar to the individual.
General dosage ranges for 3H1 have been given above.

5   *Kits comprising 3H1*

The present invention also encompasses kits containing 3H1, preferably diagnostic
kits. Diagnostic procedures using 3H1 can be performed by diagnostic laboratories,
experimental laboratories, practitioners, or private individuals. Kits embodied by this
invention include those that allow someone to conduct an assay for anti-CEA or anti-3H1

10   activity, such as any of those disclosed herein, thus detecting or quantitating these activities.

The kits of this invention comprise 3H1 in suitable packaging. The kit may
optionally provide additional components that are useful in the procedure, including, but not
limited to, buffers, capture reagents, developing reagents, labels, reacting surfaces, means for
detection, control samples, instructions, and interpretive information.

15   The following examples are provided to illustrate but not limit the present invention.

EXAMPLES

Example 1—Generation and Characterization of 3H1 Anti-Idiotype Antibody

20   The monoclonal anti-idiotype antibody producing hybridoma cell line 3H1 was
created and identified according to the following description. Aspects of both the
immunization procedure and the screening procedure were important to obtain an antibody
with the desired specificity and functionality. 3H1 was one of a number of Ab2 that were
initially produced, and was identified as the candidate with the most desirable features.

25   The immunizing antibody (Ab1) was the mouse anti-CEA monoclonal antibody 8019.
Since the responding animal was also a mouse, the Ab2 generated were expected to be
directed against idiotypic features of 8019. However, only a fraction of those would be

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directed against the 8019 paratope, an even smaller proportion would be immunogenic and capable of eliciting an Ab3, and a still smaller proportion would elicit Ab3 that cross-reacted with the tumor-associated antigen..

To render 8019 sufficiently immunogenic in an autologous species, it was conjugated
5 to the carrier KLH, and emulsified in Freund's adjuvant. It was administered repetitively into the recipient animals on an unusual schedule with only 2 weeks between doses. Five mice were immunized according to this schedule. Substantial responses arose in about 3 mice only after the fourth immunization. Responding animals were boosted with a fifth dose of 8019 i.v., spleen cells were isolated, and hybridomas were prepared separately from each animal.
10 Cloning was performed according to standard techniques.

Initial screening was conducted by immunoassay to identify the clones that reacted with 8019, but not with other target monoclonal antibodies sharing the same allotypic or isotypic determinants. A critical assay was a sandwich RIA in which 8019 is attached to a solid phase, overlayed with culture supernatant, and developed with radioiodinated 8019.
15 This assay requires the antibody in the hybridoma supernatant to be functionally bivalent, and be able to span between the capture 8019 and the developing 8019. Several clones that were idiotype specific and gave a strong signal in this assay were selected for further study.

Subsequent screening was conducted by competition assays, in which the Ab2 was required to block the binding of 8019 to CEA. This established that Ab2 recognized the
20 paratope of 8019. CEA was provided in the form of MCF-7 cells, a human breast cell tumor line expressing CEA at the cell surface. The nature of the assay requires the Ab2 to block the interaction between 8019 and the tumor antigen in its particular manner of presentation on tumor cells. At a minimum, candidate Ab2 which had passed the earlier screening tests were required to inhibit the binding of 8019 to the cells by at least 85%. There were about three
25 Ab2 that substantially exceeded the minimum, with 3H1 providing about the highest level of inhibition.

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The ultimate screening test was a determination of whether the candidate Ab2 were

capable of eliciting an Ab3 of the desired specificity when injected into a recipient.

Sufficient quantities of Ab2 were prepared from mouse ascites, and tested in mice and

rabbits. Sera from the test animals were first assayed for the presence of Ab3 in a sandwich

5   immunoassay using the same labeled Ab2 used for immunization. Sera testing positively

were then assayed for ability of the Ab3 to react against the tumor-associated antigen; namely

CEA. A semipure preparation of CEA was used to coat microtiter plates, overlayed with the

test serum in serial dilutions. and the Ab3 that bound was detected using labeled anti-

immunoglobulin. The titer of the Ab3 binding to CEA defined the "quality" of Ab2, as a

10   reflection of its capacity as an inducer of anti-CEA.

Monoclonal antibody 3H1 emerged as the anti-idiotype with the highest quality, and

is the basis for various compounds. compositions. and procedures embodied in this invention.

Materials

Carcinoembrionic antigen (CEA): Purified CEA was obtained commercially from

15   Rougler Biotech. Montreal. Canada (cat. no. 70015). Alternatively, CEA was isolated from

human liver metastasis of colonic adenocarcinoma by perchloric acid extraction and purified

twice by ion-exchange chromatography, followed by gel filtration and several steps of HPLC

chromatography. CEA obtained by this method was 100% pure, produced a single band at

180,000 m.w. by HPLC and SDS-PAGE and was immunoprecipitated as a single band by

20   horse as well as rabbit anti-CEA antibody. Two closely migrating bands of 180,000 and

200,000 m.w. were demonstrated by Western blot analysis using 8019 antibody and other

murine mAb anti-CEA. The purified CEA was used for ELISA experiments with mouse and

rabbit polyclonal Ab3 sera, described supra.

Other experiments were generally conducted using a semipurifed extract from human

25   adeoncarcinoma cells. This was prepared by perchloric extraction followed by extensive

dialysis. The presence of CEA in the extract was confirmed by SDS-PAGE, followed by

immunoprecipitation with mAb 8019.

Antibody: The hybridoma cell line producing monoclonal antibody 8019 was

obtained from the American Type Culture Collection (ATCC, Rockville, MD). The antibody

was originally described as an IgM κ, but during recloning a spontaneous switch mutant

appeared, and our 8019 is an IgG1 κ. The specificity of 8019 was reconfirmed by

5   immunoperoxidase staining and flow microfluorimetry analysis using cells expressing CEA.

Monoclonal antibody 1E3 mAb (IgG1κ; specific for human mucinous ovarian carcinoma)

and other monoclonal and myeloma mouse immunoglobulins were used as controls in

various experiments herein described.

Ascites of 8019 hybridomas and other cell lines were prepared by injecting

10   individual pristane-primed mice i.p. with 2-10 x 10^6 viable cells. The IgG fraction was

isolated from ascites by 45% saturated ammonium sulfate precipitation and subsequent

chromatography on Protein A Sepharose(TM) CL-4B (Ey et al. (1978) Immunochemistry

15:429). The purity of the isolated IgG was checked by immunodiffusion,

immunoelectrophoresis, and high pressure liquid chromatography (HPLC) fractionation.

15   *Preparation of F(ab')₂ fragments of 8019:* The F(ab')₂ fragments were prepared by

standard pepsin digestion (Parham (1983) J. Immunol. 131:2895). Briefly, the IgG fraction

from the 8019 ascites was dialyzed against 0.1 M citrate buffer, pH 3.5, and digested with

pepsin (25 μg/mg IgG) at 37°C for 8 h. After cleavage, the pH was adjusted to 7.0 with 3.0

M tris buffer, pH 8.6, and the solution was dialyzed against phosphate-buffered saline (PBS)

20   in the cold. The digest was separated by HPLC using a Sepharose 6 column. The purity of

the isolated F(ab')₂ was determined by immunodiffusion and by reaction with anti-isotype

reagents in a standard ELISA.

Coupling of antibody with KLH: 8019 was coupled to keyhole limpet hemocyanin

(KLH) according to a method described by Maloney et al. (1985; Hybridoma 4:191).

25   Antibody stock solution (1 mg/ml) was mixed with KLH (1 mg/ml) in PBS in the presence of

freshly diluted glutaraldehyde solution (final concentration 0.05%). The mixture was rotated

end-over-end for 1 h at room themperature, and then dialyzed exhaustively against PBS at

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4°C. *Immunization of syngeneic BALB/c mice:* BALB/c females were immunized four times

over a perioud of 2 months. The first injection was given i.p. using 100 µg of 8019,

emulsified in complete Freund's adjuvant. The next two injections were given with 100 µg of

8019 coupled to KLH in incomplete Freund's adjuvant, either s.c. or i.p. Mice were bled

5   from time to time, and sera were checked for anti-Id activity by ELISA in a binding assay by

using F(ab')$_2$ fragments of 8019 and normal pooled BALB/c mouse serum IgG as control.

Three days before the fusion, the mice were boosted i.v. with 8019 in PBS.

Production of anti-idiotype hybridomas

The fusion partner used to produce the hybridoma lines was the mouse non-secretory

10   myeloma cell line P3-653, ancestrally related to P3X63Ag8.653, available from the ATCC as

No. CRL-1580. Established human cell lines were cultured in RPMI 1640 supplemented

with 5% fetal calf serum as described elsewhere (Seon et al. (1984) J. Immunol. 132:2089).

Hybridomas were produced essentially following the method of Oi and Herzenberg

((1980) "Selected Methods of Cellular Immunology", Mishell & Shiigi eds., Freeman Publs.,

15   at 351-372). Speen cells from immunized mice were mixed with P3-653 cells at a ratio of

1:1 to 1:10, in the presence of 50% polyethylene glycol (PEG, mw ~4500). Fused cells were

then washed and cultured. Hybrids were selected using hypoxanthine-aminopterin-thymidine

media.

Initial selection of anti-idiotype antibody (Ab2) secreting hybridoma clones:

20   Initial screening of the hybridoma clones was performed by RIA and ELISA. The

ELISA was conducted by coating microtiter plate wells with 8019 antibody (or control) at

500 ng/well. After incubating overnight at 4°C, the plates were blocked with 1% bovine

serum albumin (BSA) in PBS. 100 µl of hybridoma culture supernates or 20 x concentrate

was incubated in the well for 4 h at room temperature. After washing with PBS, the plates

25   were further incubated for 4 h at room temperature or overnight at 4°C with alkaline

phosphatase-labeled anti-isotype reagents, and developed with the substrate. Because the

ELISA detecting reagents were anti-mouse immunoglobulin, the 8019 used to coat the plates

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was an F(ab')$_2$ fragment. The ELISA assay is useful in identifying the class and subclass of specific antibody. Generally, antibody of certain IgG subclasses is desired because it is stable, easily purified by protein A chromatography, and may have useful effector functions.

Hybridoma supernatants were also tested in a sandwich RIA. Purified 8019 was

5   radioiodinated by the chloramine T method (Hunter (1970) Proc. Soc. Exp. Biol. Med. 133:989). 8019, or control antibody (monoclonal antibodies of various isotypes and unrelated specificities, and BALB/c normal IgG) was coated onto PVC plates at 500 ng/well. Generally, intact antibody was used. After incubating overnight at 4°C, the plates were blocked with 1% BSA in PBS. Coated plates were incubated with serial dilutions of

10   hybridoma supernatant for 4 h, and developed using ~50,000 cpm of [125]I-8019. The RIA assay is a more stringent specificity test for the antibody, and also requires that the antibody be able to span between two 8019 molecules.

A number of monoclonal Ab2 secreting cell lines emerged from these screeining assays with the desired properties. Amongst them was monoclonal antibody 3H1.

15   Confirmation that Ab2 are specific for 8019 idiotype

Idiotype specificity of Ab2 was confirmed by direct binding to Ab1. Various purified Ab2 were labeled with ^{125}I, and tested for binding to plates coated with a panel of monoclonal anti-TAA Ab1. Results for an experiment using [125]I-3H1 are shown in Figure 5. The results are presented in mean cpm (n=3, S.D. < 10%). 3H1 bound almost exclusively to

20   8019; there was virtually no cross-reactivity with any of the other Ab1 tested, with a single exception: Minor cross-reaction with anti-CEA antibody RWP 1.1 (IgG2b, κ) that recognizes a related (possibly overlapping) epitope on CEA.

Specificity for the 8019 idiotype was further established in competition experiments. ~25,000 cpm of various labeled Ab2 was mixed with different members of a panel of

25   unlabeled competitors comprising Ab2, Ab1, and other mouse immunoglobulins. The Ab2 was then tested for binding to 8019 coated plates. Results are shown in Table 1 (mean cpm, n=3, S.D. < 10%). Greater than 90% inhibition was obtained using 250 ng of unlabeled 3H1

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or 8019 as competitor. Virtually no inhibition was obtained, up to a concentration of 5 μg,

using the other immunoglobulins as potential competitors, except for the related Ab1

antibody RWP 1.1.

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<u>TABLE 1</u>

Inhibition of Id-anti-1d binding*

Inhibitor	cpm Bound	Percent Inhibition
None	11,995	0
3H1 (Ab2), 0.125 μg	439	97
8019 (Ab1), 0.200 μg	861	95
RWP 1.1, 5 μg (anti-CEA)	1,842	85
1E3, 5 μg (anti-iso, allotype)	11,755	2
Mc-10, 5 μg (anti-iso, allotype)	12,085	0
F36/22, 5 μg (anti-iso, allotype)	11,558	4
3F3, 5 μg (anti-CEA)	10,955	8
ZCE, 5 μg (anti-CEA)	12,033	0
31C5A4, 5 μg (anti-CEA)	11,800	1
D-14, 5 μg (anti-CEA)	12,075	0

<u>Screening for anti-idiotypes directed against the 8019 paratope</u>

To determine whether the Ab2 were directed against the paratope of 8019, the Ab2 were used to compete for the binding of radiolabeled 8019 to CEA. This was performed two ways: (1) plate-binding assays were conducted using the semipurified CEA extract; (2) cell binding assays were conducted using LS174T cells, a human colon cancer cell line expressing CEA as a membrane constituent

Plate-binding assays were coated by incubating plates with 100 μl of the perchloric acid solubilized semipurified CEA Ag extract (0.1 mg protein/ml) overnight at 4°C. LS174T cells were grown as confluent monolayer in 96-well tissue culture plates. Various dilutions of the test Ab2 (either culture supernatnat or purified antibody) were mixed with the labeled 8019, and then added to the coated plate or cultured cells. Percent inhibition of the assay was calculated according to the formula:

$$\% \ inhibition = 1 - (\frac{R_T - R_C}{R_{MAX} - R_C}) \ x \ 100$$

where R_T is the average cpm of the experimental well with inhibitors; R_C is the average

background cpm; and R_{MAX} is the average maximum binding without any inhibitors.

Figure 6 shows results of this type of experiment. conducted using 3H1 as the model

competitor in the plate-binding assay. 3H1 inhibited the binding of labeled 8019 to the CEA

5   at amounts as low as 25 ng. Purified antibody 4EA2 (an IgG1,k of unrelated specificity) was

used as a negative control, and demonstrated no inhibition. In a related experiment, 3H1 was

not able to inhibit the binding of another anti-CEA antibody (D14) to the CEA-coated plates.

Confirmation of the binding specificity

For the most promising Ab2. confirmation experiments were conducted to confirm

10   the specificity of binding to 8019. in which the roles in the competition assay were reversed.

About 40.000 cpm of ^{125}I-8019 was coincubated with a semipurified preparation of

CEA Ag, or else with a nonrelated glycoprotein Ag that does not react with 8019

(Bhattacharya et al. (1982) Cancer Res. 42:1560). The antibody-Ag mixture was added to

Ab2-coated plates (500 ng/well), and the ability of CEA to inhibit the binding was

15   determined. The amount of Ab2 was non-limiting with respect to the amount of 8019 that

could bind, and was therefore a sensitive indicator for small amounts of competing CEA.

Figure 7 shows the results of a typical experiment. 2.5 μg of semipurified CEA

inhibited the binding of a3H1 to iodinated 8019 by 50%. The unrelated glycoprotein even at

higher concentration did not inhibit binding. This suggests that 3H1 is a binding site-specific

20   anti-Id.

Antibody-producing clones testing positively in the screening tests described so far

were used to prepare mouse ascites as a source of Ab2. The Ab2 were purified by

chromatography using Protein A and Protein G affinity resins by standard techniques.

Screening for anti-idiotypes capable of eliciting a tumor-specific immune response

If the Ab2 behaves as a network antigen, then it should induce the production of Ag-

25   specific Ab3 in the absence of exposure to Ag in a genetically unrestricted way and across

species barriers. Accordingly, Ab2 that had passed previous screening tests were screened

further in immunization experiments. The objective is to identify the candidates that can

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elicit Ab3 sharing idiotypes with Ab1, and exhibiting a similar binding specificity for the tumor-associated antigen.

.For each Ab2 to be tested, a minimum of 5 BALB/c mice and two New Zealand white rabbits were immunized. For immunization of mice, the Ab2 was conjugated to KLH. 50μg

5   was injected, and the mice were bled periodically to test the response. 500μg was injected per rabbit, emulsified in complete Freund's adjuvant on day 0, in incomplete Freund's adjuvant on day 14, and in saline (i.m.) during the next 2 months. The rabbits were bled 14 days after the last injection.

Anti-CEA activity was measured by ELISA (see geenrally Engvall et al. (1972) J.

10   Immunol. 109:129). Various dilutions of test sera were incubated in CEA coated wells, and antibody bound was detected with enzyme-linked anti-immunoglobulin appropriate for the species. This assay requires the antibody to bind the original tumor-associated antigen, and establishes that at least a portion of the Ab3 induced by immunizing with the anti-idiotype is tumor antigen specific. The level of CEA-specific Ab3 was titered by serial dilution, and

15   defined the "quality" of the immunizing Ab2. Sera from mice and rabbits immunized with an unrelated monoclonal antibody (4EA2) was used as a negative specificity control

The 3H1 monoclonal antibody emerged as having the highest quality amongst the candidates tested.

As shown in Figure 8, Ab3 present in the sera of mice immunized with 3H1 was

20   specific for insolubilized CEA. All immunized mice (six in two groups) developed anti-CEA antibody as measured by ELISA. Control sera from preimmune mice or mice immunized with an unrelated Ab2-KLH (4EA2) did not show binding to pure CEA. In a parallel experiment, the binding of the same antisera was compared on a plate coated with unrelated ovarian tumor glycoprotein. The maximum binding obtained in each case was between 0.3 to

25   0.4 OD, the same as obtained with PBS-BSA control.

In a related experiment, the binding of Ab3 to cultured human colon carcinoma LS174–T cells were tested in an indired immunofluorescence assay and flow cytometry. As

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shown in Figure 9. Ab3 containing sera from 3H1-immunized mice showed distinct

binding (B) that was similar to the binding pattern obtained with 8019 (Ab1) (A). No

significant binding was obtained with human B cell lymphoma cells which do not express

CEA (Figure 9D).

5   Confirmation that the Ab3 elicited by 3H1 had the desired specificity

Since the therapeutic objective of 3H1 lies in its ability to elicit a response reactive

against the tumor associated antigen, the specificity of the Ab3 obtained was confirmed in a

number of subsequent experiments.

The rabbit and mouse Ab3 antisera were depleted of anti-isotype and anti-allotype

10   activity for use in the specificity experiments by passing over an adsorbant made by coupling

immunoglobulin fractions of BALB/c mouse serum coupled to 4B. Adsorption was repeated

until no anti-isotype or anti-allotype activity could be detected by immunodiffusion.

Adsorbed Ab3 containing sera were diluted with PBS containing 1% BSA, 0.05% Tween 20,

and used in specificity determination without any further purification.

15   Spleen cells from mice immunized with 3H1 were used to generate monoclonal Ab3

producing cell lines, using similar hybridoma technology as described earlier.

Inhibition assays: To determine whether Ab3 sera compete with Ab1 for binding to

human colon carcinoma cells, the binding of radioiodinated 8019 to confluent monolayers of

LS174-T cells was tested for inhibition in the presence of different Ab3 sera and Ab1.

20   For direct binding assay between Ab1 and 3H1, purified 3H1 was used to coat plates

(155 ng/well), and the binding of radiolabeled 8019 to 3H1 was tested in the presence of

different Ab3 and Ab1. Percent inhibition of the assays were calculated according to the

formula described above.

Sera from syngeneic mice immunized with 3H1, at 1/10 dilution, inhibited binding or

25   iodinated 3H1 (Ab2) to Ab1 by 90%. No inhibition by preimmune sera or sera from mice

immunized with unrelated Ab2, 4EA2-KLH was observed. Although stearic hindrance by

Ab3 binding cannot be excluded in these assays, the results suggest the presence of Ab3

antibodies that share idiotopes with Ab1 (8019). The antisera from rabbits 729 and 730. immunized with 3H1, at 1/10 dilution. inhibited binding or iodinated 8019 to Ab2 by 88 and 57%, respectively. No significant inhibition was obtained with preimmune rabbit sera.

5   If Ab3 has a similar binding site as Ab1. it should compete with Ab1 for binding to CEA as expressed by the human carcinoma cell line LA174-T. A fixed amount of radiolabeled 8019 was coincubated with different dilutions of rabbit Ab3 sera or Ab1 preparation and LS174-T cells (Figure 10). Twenty ng of purified 8019-IgG1 (Ab1) inhibited binding by 50%. whereas the rabbit sera to 1/10 dilution produced 47 and 49% inhibition respectively for rabbit 729 and 730. This indicated that polyclonal rabbit Ab3 sera

10   bind to the same Ag as Ab1 and therefore contain some antibody molecules with Ab1 properties.

       Western blot analysis: The semipurified CEA extract was separated by standards SDS-PAGE in 7.5% gel under nonreducing conditions without ß-mercaptoethanol. After electrophoresis the gel was transblotted to nitrocellulose filters according to the procedures to

15   Towbin et al. ((1979) Proc. Natl. Acad. Sci. USA 76:4350). The filter strips were blocked with PBS- 1% BSA and then incubated separately with 8019, polyclonal rabbit Ab3 sera, control rabbit Ab3 sera against unrelated Ab2, as well as monoclonal Ab3 culture supernatant. After incubation, the filter strips were washed with PBS and incubated with goat anti-mouse Ig or goat anti-rabbit Ig-alkaline phosphatase labeled reagents. The filter

20   strips were again washed and the reaction was developed with the reagents BCIP and NBT supplied for an immunoblot kit (Bio-Rad Laboratories, Richmond, CA).

       It has been shown that mAb 8019 specifically immunoprecipitates the 180,000 m.w. CEA by SDS-PAGE analysis (Mitchell (1980) Cancer Immunol. Immunother. 10:1). To confirm that the Ab3 induced by 3H1 was specific for the CEA molecule, semipurified

25   extract of CEA was separated by SDS-PAGE and transblotted to nitrocellulose filters. One filter strip (Figure 11, lane 2) was stained with buffalo black. There were two overlapping bands at the 180,000 m.w. region (CEA) and one major band at the 50,000 m.w. region

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(normal cross-reacting Ag) and a few minor low m.w. bands. The remaining filter strips

were then incubated with mAb 8019, rabbit Ab3 sera, and rabbit sera immunized with the

unrelated isotype-matched Ab2β 4EA2 (a negative control). The reaction was developed by

the ELISA assay as described above. Antibody 8019 (Figure. 11, lane 3) and rabbit Ab3

5   (lane 4) immunoprecipitated only molecules with a molecular mass of 180,000 Da from this

complex mixture. The materials that were not precipitated by mAb 8019 or rabbit Ab3 sera

contained a wide range of lower m.w. CEA-related Ag. There was no reactivity with

preimmune (Figure. 11, lane 5) or control sera (lane 6). The Western blotting analysis

confirmed the specificity of mAB 8019 and the reactivity of rabbit Ab3 with 180,000 m.w.

10   CEA.

Figure 12 is a similar experiment conducted with mouse sera. The Ab3 elicited in

mice immunized with 3H1 identified the same 180,000 m.w. form of CEA in the Western

blot.

Immunoperoxidase staining of tissue sections with Ab1 and Ab3: The reactivities of

15   monoclonal Ab1 and Ab3 (both polyclonal and monoclonal) were compared on surgical

specimens of normal colon and colonic adenocarcinomas by a very sensitive staining method

(biotin-streptavidin reagents, Vector, Burlingame, CA) as described in detail by Viale et al.

((1989) J. Immunol. 143:4338). All sections were counterstained with Meyer's hematoxylin.

Pertinent specificity tests were performed, including block of the endogenous peroxidase,

20   omission of the first layer, or substitution of nonimmune homologous serum for the specific

antiserum and P3-653 myeloma culture supernatant for the Ab3 culture supernatant.

The reactivity of 8019 were compared with that of Ab3 (both polyclonal and

monoclonal) on normal colon and colonic tumor specimens. The pattern of reactivity of Ab3

on both normal and malignant colonic tissues was almost identical to that obtained with Ab1

25   (Figure 13). There was no reaction with normal colonic mucosa, but 8019 and all the Ab3

reacted intensely with colonic tumors. The staining was apical in gland-like structures and

granular (cytoplasmic) in less differentiated areas. There were subtle differences between the

staining patterns obtained with 8019 (an IgG1, κ) and the monoclonal Ab3 (an IgM, κ).
Reaction with 8019 resulted in the staining of tumor cells as well as secreted mucinous
materials, whereas reaction with monoclonal Ab3 resulted in the staining of tumor cells with
no staining of secreted mucin. (Figure 14).

5   *Tests of cellular immunity:* Additional experiments may also be conducted to
demonstrate that the animals immunized with 3H1 also have a CEA-directed cellular immune
response. Spleen cells from mice immunized with 3H1 may be used in a T-cell proliferation
assay. The spleen cells arecultured for 5 days in the presence of semipurified CEA, and then
pulsed with [^{3}H]thymidine. Greater uptake in cells from 3H1 immunized animals than with

10   controls is consistent with the presence of an idiotype-specific cellular immune response.
Immunized rabbits may also be tested for DTH skin reactions against semipurified
preparations of CEA or purified CEA. T cell cytotoxicity assays may also be conducted, as
described elsewhere in this disclosure.

A schematic showing an anti-idiotype network based on 3H1 is shown in Figure 15.

15   **Example 2—Cloning and Sequencing of 3H1 cDNA**

Unless otherwise specified, all cloning techniques were essentially as described by
Sambrook et al. (1989) and all reagents were used according to the manufacturer's directions.

cDNA cloning and sequence determination of the variable regions of 3H1

To sequence the V$_H$ region, total RNA was isolated from 1x10^7 3H1 hybridoma cells.

20   Yield of total RNA was about 100 μg,. mRNA was prepared by passage through two-cycles
of chromatography of oligothymidylate-cellulose columns. The yield of mRNA was about
10μg. First strand cDNA was synthesized using SuperScript Preamplification kit
(GIBCO/BRL). The DNA fragment encoding the V$_H$ of 3H1 was then amplified by PCR
using the 5'-primer GGGAATTCATGRAATGSASCTGGGTYWTYCTCTT and the 3'-

25   primer CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG (I=inosine, R=A or G,
Y=C or T, K=G or T, S=C or G, W=A or T) corresponding to sequences of the leader (signal
peptide) region amino acids -20 to -13, and the gamma constant region amino acids 126 to

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119. In addition, the 5'-III site provided an alternative cloning strategy (Novagen, Madison

Wisconsin). The fragment of cDNA amplified was subcloned into pT7 plasmid and

NovaBlue competent cells were transformed using a protocol provided by the supplier

(Novagen). Recombinant colonies were picked up by color selection and plasmid DNA was

5   prepared. The DNA sequence of the double stranded plasmid was determined by Sequenase

Version 2.0 kit (USB, Cleveland, Ohio). The sequence of the DNA insert in the plasmid was

determined from both orientations using T7 promoter primer

(TAATACGACTCACTATAGGG) and U-19 primer (CTTTTCCCAGTCACGACGT). At

least 8 clones were picked for sequence determination. The sequence of the 3H1 light chain

10   was similarly determined. The forward primer for the light chain was 5' -

ACTAGTCGACATGGTRTCCWCASCTCAGTTCCTTG and the reverse primer was 5' -

CCCAAGCTTACTGGATGGTGGGAAGATGGA, corresponding to -20 to -12 amino acids

of the leader sequence and 122 to 116 of the constant region of the mouse kappa chain.

In order to minimize the error rates in PCR amplification, pfu DNA polymerase

15   (Stratagene, San Diego) was used for amplification in all subsequent experiments. Mutant

frequency with this thermostable DNA polymerase is 1/10 compared to Taq DNA

polymerase.

Verification of the cDNA clone by amino acid sequence

Although 3 clones that we picked all had the same sequence, we felt it necessary to

20   confirm that the isolated cDNA was indeed that of 3H1. Fifty μg of purified 3H1 antibody

was diluted with sample loading buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% glycerol,

0.1% β-mercaptoethanol) and heated to 100° C for 3 minutes. The denatured protein was

loaded onto a 7.5% polyacrylamide gel (BioRad Miniprotean II Dual Slab Cell) containing

SDS and subjected to electrophoresis at 200 V for 1 hour. Proteins in the gels were

25   transferred to polyvinylidene difluoride (PVDF) membranes by the procedure described by

Twobin et al. ((1979) Proc. Natl. Acad. Sci. USA. 78: 4350-4354) at 150 mA overnight.

The transfer buffer contained 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. The

membranes were stained by quick dipping in 0.1% Coomassie Brilliant blue in 50%

methanol-50% acetic acid, followed by washing in a solution containing 40% methanol plus

10% acetic acid. After drying the membrane at room temperature, the stained heavy and light

chain bands were excised with a clean razor blade. The proteins on the membrane slices

5   were subjected to N-terminal microsequencing by automated Edman degradation using an

Applied Biosystem Model 477A protein sequencer employing pulsed-liquid chemistry and

on-line phenyl-ethiohydantion amino acid identification. Each protein was subjected to 10-

15 degradative cycles and the converted cleavage products from each cycle were analyzed by

reverse-phase HPLC. The sequencing was done by Macromolecular Structural Facility of the

10   University of Kentucky. The sequence of the peptide was (Glu)

ValGlnLeuGlnGlnSerGlyProGluLeuValLysProGly. Except for the first Glu whose identity

was uncertain, 14 amino acid residues of the peptide matched exactly with the amino acids 2-

15 of 3H1 heavy chain. This confirmed that the cDNA clone picked was that of the 3H1

heavy chain.

15   cDNA (SEQ ID NO:1) and derived amino acid sequence (SEQ ID NO:2) of the light

chain variable region of 3H1 is shown in Figure 1. cDNA (SEQ ID NO:3) cDNA and

derived amino acid sequence (SEQ ID NO:4) of the heavy chain variable region of 3H1 is

shown in Figure 2.

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Example 3—Analysis of Immune Response Elicited by 3H1 in Non-Human Primates

Cell Lines

     The human colon carcinoma cell line LS174-T, which expresses CEA at high density,

5   was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine

and penicillin and streptomycin and used for the detection of anti-tumor responses. The

human melanoma cell line M21/P6 (kindly provided by Dr. Ralph Reisfeld, Scripps Research

Institute, La Jolla, California), and the T-cell line MOLT-4, both of which are CEA negative,

were grown in the same medium and were used as negative controls.

10   _Antibodies_

     3H1 was obtained as described in Example 1. The mAb2, 11D10 (IgG1,κ) is a

murine anti-Id mAb which mimics the human milk fat globule (CEA) (Mukerjee et al. (1992)

FASEB J 6:A2059) and was used as a control.

Preparation of 3H1 for Immunization

15   We immunized monkeys with 3H1 precipitated with alum prepared as follows.

Herlyn et al. (1987) _PNAS_ 84:8055-8059.

     To 5 mg aliquots of purified mAb anti-Id (3H1), 1 ml of 2% Alu-Gel S (Serva Fine

Biochem, Inc., Garden City, Long Island. NY) was added. The volume was then adjusted to

10.0 ml with D-PBS and the mixture incubated on a vortex for one hour at room temperature.

20   The mixture was then centrifuged at 2000 rpm at 24°C for 10 minutes. The amount of mAb

bound in the gel layer was determined by measuring spectrophotometrically the amount of

unbound antibody in the supernatant. The Alu-Gel precipitated antibody was stored at 4°C

until use. These procedures were performed aseptically in a laminar flow hood and the final

product was sterile and clearly labeled as anti-Id 3H1 Alu-Gel and aliquoted into pyrogen-

25   free, sterile glass vials.

Immunization of Monkeys

Cynomolgus monkeys were immunized with alum precipitated anti-Id 3H1 as well as with control alum precipitated anti-Id 11D10 (specific for CEA). Monkeys were housed at the White Sands Research Institutes, Alamogordo, New Mexico. A pair of male and female

5 monkeys, weighing 3-4 kg, was immunized with either 2 mg of 3H1 or 11D10 intracutaneously at four different sites on day 0, 14, 28 and 42 respectively. Only two monkeys were used for each anti-Id (Ab2) at a single dose for financial reasons. The 2 mg dose was selected based on previous pre-clinical (Chattopadhyaya et al. (1992) PNAS 89:2684-2688) and clinical studies (Herlyn et al. (1987) PNAS 84:8055-8059; Mittelman et

10 al. (1992) PNAS 89:466-470) with different anti-Id vaccines. Blood samples were collected before immunization and 10 days after each immunization.

Toxicity

The induction of Ab3 responses in monkeys did not cause any apparent side effects in animals. Only mild local swelling and irritation were observed at the injection site as a result

15 of multiple immunizations. The monkeys were routinely checked by physical examinations and weight measurements.

Purification of anti-anti-Id antibody (Ab3) from hyperimmunized monkey sera

Twenty milliliters of hyperimmune serum were passed over an immunoabsorbent column consisting of immunizing anti-Id immunoglobulin (3H1-IgG1) coupled to Sepharose

20 4B. Anti-anti-Id antibodies (Ab3) were eluted with 0.1 M glycine-hydrochloric acid buffer (pH 2.4) and neutralized to pH 7.0 with 3M Tris. The eluted antibody was then passed over an immunoabsorbent column consisting of isotope-allotype matched murine immunoglobulin coupled to Sepharose 4B to remove anti-isotope and anti-allotypic reactivities. Antibody that passed through was concentrated and used as purified Ab3. The isotope of Ab3 was

25 determined by ELISA using human anti-isotope specific reagents (Tago Inc., Burlingame, CA).

Development of Humoral Immunity Induced by Immunization with Alum-Precipitated 3H1

(a)   Specific Ab3 response to 3H1

Sera obtained from immunized monkeys 10 days after the fourth immunization were

tested for the presence of anti-anti-Id antibodies (Ab3). For these assays, the sera were pre-

5   treated with normal mouse immunoglobulin (500 μg/ml) to block anti-isotypic and anti-

allotypic reactivities and then checked for the presence of (Ab3) by reaction with the

immunizing anti-Id (3H1) coated onto microtiter plates, by radioimmunoassay (RIA). Ab3 at

a 1:40 dilution was incubated with anti-Id MAb 3H1 or 11D10, coated on the microtiter plate

and then reacted with ^{125}I-labeled 3H1 or 11D10 (50,000 cpm) in a sandwich assay. The

10   results are expressed as bound cpm in a sandwich assay (A). The results are presented as

mean cpm (n=3). The SD of the data was <10%. For the binding inhibition assay between

Ab2 and Ab1, purified Ab1 8019 was used to coat the plate (250 ng/well) and the binding of

radiolabeled Ab2 to Ab1 was tested in the presence of different dilutions of Ab3 sera. The

sera were preincubated with normal mouse IgG prior to the assay. The results are expressed

15   as percent inhibition at a dilution of 1:40. Unrelated Ab2 11D10 was used as the control.

After washing, the anti-antibody reaction was tagged using ^{125}I-labeled anti-Id reagent in a

homogeneous sandwich RIA. Pre-immune sera and sera from monkeys immunized with

control Ab2, 11D10 were also used in these assays. In addition, ^{125}I labeled monoclonal Ab2

11D10 was used as control. The results are shown in Table 1.

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Table 1

Analysis of Monkey Anti-Anti-Id Sera Generated With Anti-ID mAb 3H1

Assay	Plate Coated With	^{125}I Labeled Anti-ID	Sample				
			Ab3 Sera		Pre-Immune Sera	Ab3 Sera (Control)	
			PRO 541	PRO 667		PRO 723	PRO 872
A. Sandwich RIA	3H1 (Ab2)	3H1	16,381	20,143	309	926	511
	11D10 (Unrelated Ab2)	3H1	382	410	349	1,074	978
	3H1 (Ab2)	11D10	887	1,049	167	301	532
B. Inhibition	8019(Ah1)	3H1	88	84	3	<10	<10

pa-53830

Serial dilutions of Ab3 sera from monkeys (PRO 541 and PRO 667) immunized with

3H1 bound specifically to the immunizing Ab2, 3H1, with minimal reactivity with unrelated

Ab2, 11D10. Monkey Ab3 sera also inhibited the binding of radiolabeled Ab2 to Ab1 over

80% even at a dilution of 1:40 (Table 1). There was no inhibition with pre-immune sera or

5   sera obtained from monkeys (PRO 723 and PRO 872) immunized with the unrelated Ab2,

11D10.

The kinetics of the Ab3 response are shown in Figure 16 using sera from monkey

PRO 667 demonstrating inhibition of the binding of radiolabeled Ab2 to Ab1. Similar

reactivity was seen with sera from monkey PRO 541. These results indicate that monkey

10   Ab3 sera share idiotypes with the Ab1 (8019).

(b)   Idiotope analysis of Ab3

If a positive reaction was obtained in (a) above, Ab3 sera from those monkeys were

checked for their ability to inhibit the binding of ^{125}I-labeled 3H1 to 8019 (Ab1) bound to

microtiter plates or vice versa (inhibition of the binding of radiolabeled 8019 to 3H1 on the

15   plate). An unrelated Ab1-Ab2 system (BrE1-11D10) was used as control (Mukerjee et al.

(1992) FASEB J. 6:A2059). For this experiment, purified 8019 (Ab1) was used to coat the

plate (250 ng/well) and the binding of radiolabeled 3H1 (~50,000 cpm) to 8019 was tested

for inhibition in the presence of different dilutions of Ab3 sera. In a parallel control

experiment, an unrelated Ab1-Ab2 system (mAb BrE1-11D10) was used as a control.

20   This demonstrated that Ab3 in monkey sera share idiotopes with 8019 (Ab1). This

inhibition assay of Ab1 - Ab2 binding by Ab3 sera also demonstrated that Ab3 is a true anti-

anti-Id.

(c)   Induction of Anti-CEA Antibody Response

Microtiter plates were coated with pure CEA (Rougier Biotech, Montreal, Canada)

25   and reacted with the Ab3 sera, obtained after the fourth immunization at different dilutions

by ELISA. A 96-well microtiter plate was coated with 100 µl of the purified preparation of

CEA (2 µg of protein per ml) per well and incubated overnight at 4°C. The solution was

removed from the plate by suction. and the plate was blocked with 1% BSA in PBS to

saturate protein-binding sites. After incubation for 1 hour at room temperature, the solution

was removed, and the wells were washed three times with PBS. Samples of diluted serum

(50 μl), mixed with 50 μl of 0.05% Tween 20/1% BSA in PBS, were added in duplicate and

5   incubated overnight at 4°C. After washing the plate, 0.1 ml of enzyme-labeled antibodies

diluted 1/1000 in 0.05% Tween 20/1% BSA in PBS was then added and incubated at room

temperature for 4 hours. The plates were again washed and developed with 0.1 ml of

phosphatase substrate dissolved in diethanolamine buffer (50 mg of substrate per 50 ml of

buffer). The absorbance at 405 nm was read on an ELISA Reader. The sensitivity of the

10   assay was greater than 0.1 ng of antibody detected per well. Pre-immune sera and sera

obtained from monkeys immunized with unrelated Ab2 11D10 were used as a control. The

unrelated antigen HMFG was also used as a control in this assay.

The Ab3 sera from monkey PRO 541 bound specifically to purified CEA (Figure 17),

while control sera from pre-immune monkeys or monkeys immunized with unrelated Ab2,

15   11D10, did not show appreciable binding to CEA. In parallel experiments, the same Ab3

from monkey PRO 541 was compared on a plate coated with control CEA antigen and were

negative. The kinetics of the anti-CEA response from monkey PRO 667 are shown in

Figure 19.

To determine the reactivity with cell-surface CEA, LS174-T cells were tested by

20   immune flow cytometry. CEA positive LS174-T cells (1×10^6 per well) were reacted with

Ab1 (8019) and Ab3 at 100 μl at 4°C for 60 minutes. After washing, the cells were incubated

with either goat anti-mouse or goat anti-human F(ab')$_2$ IgG-FITC labeled antibody (Tago

Inc., Burlingame, CA) for 30 minutes at 4°C. They were then washed twice, fixed in 2

percent paraformaldehyde, and analyzed by immune flow cytometry (FACS STAR, Becton

25   Dickinson, San Jose, CA). Antigen negative MOLT-4 cells and melanoma cells M21/P6 (not

shown) were used as controls in this assay.

As shown in Figure 28. Ab3 from 3H1 immunized monkeys showed distinct binding (Figure 20–1) that was similar to the binding pattern obtained with Ab1. Significant binding was not obtained with MOLT-4 cells which do not express CEA (Figure 20–2). There was also no binding with melanoma cells.

5   We then compared the reactivities of Ab1 (8019) with that of monkey purified Ab3 (50 μg/ml) by a sensitive immunoperioxidase assay on human colon tumor and normal colon specimens. The Ab3 antibodies were purified from sera as described above. The method (biotin-streptavidin reagents. Vector. Burlingame. CA) has been described in detail elsewhere (Battacharya-Chatterjee (1990) and (1991)). All sections were counterstained with Meyer's

10   hematoxylin. Pertinent specificity tests were performed, including block of the endogenous peroxidase. omission of the first layer. or substitution of nonimmune homologous serum for the specific antiserum and P3-653 myeloma culture supernatant as the control.

The pattern of reactivity of Ab3 (Figure 20–1) on the colon cancer specimen was identical to those obtained with Ab1 8019 (Figure 20–2) whereas there was no reactivity with

15   unrelated monkey Ab3 (Figure 20–3). Also there was no reactivity of either Ab3 (Figure 20–4) or Ab1 with control normal colon section. Reactions with Ab1 or purified Ab3 (Figures 20–1. 20–2) resulted in the staining of both tumor cells as well a secreted mucinous materials. The staining was apical in gland-like structures and granular (cytoplasmic) in less differentiated areas. Monkey Ab3 was further tested on normal stomach, duodenum, cecum,

20   smooth and striated muscle tissues and found negative.

To further exclude the possibility that the Ab3 antibodies react with a minor contaminant in the purified CEA preparation, or that these antibodies stain a non-CEA membrane protein on tumor cells, we identified the molecular species precipitated by the monkey purified Ab3. Purified CEA was labeled with ^{125}I by the Chloramine T-method and

25   reacted with purified Ab3 (10 μg) or Ab1 (10 μg) or unrelated control Ab3 from monkey immunized with Ab2 11D10 (10 μg) or PBS-BSA control, previously adsorbed onto protein G-Sepharose beads. After washings. the antigen-antibody coated beads were analyzed by

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SDS-PAGE according to the method of Laemmli ((1970) Nature 227:680-685) and

radioautographed. As seen in Figure 21, the radiolabeled material was precipitated by the

monoclonal 8019 (Ab1) and the monkey Ab3. The molecular weights by SDS-PAGE was

identical.

5   (d)   Epitope analysis of Ab3 using LS174-T cells

         To demonstrate that Ab3 generated in monkeys and Ab1 (8019) bind to the same

antigenic determinant, inhibition of 8019 binding to the antigen positive tumor cell line

LS174-T cells or CEA by purified Ab3 was checked by RIA as described (Bhattacharya-

Chatterjee et al. (1990) and (1991)). This assay was done in disposable microfold 96-well

10   microfilter-coated plates. The plate was first treated with 10% FCS and 1% BSA in PBS. In

a typical assay, triplicate 50-μL aliquots of various dilutions of purified Ab1 or Ab3

preparation and 5x10^5 viable cells in 50 μl of PBS were co-incubated with a fixed amount of

radiolabeled Ab1 (~50,000 cpm) in individual wells for 2 hour at room temperature with

continuous shaking.

15   After incubation, the plate was washed three times with PBS/1% BSA by suction.

The radioactivity in the washed filter paper was determined in a K-ray spectrometer.

         Percent inhibition of the assay was calculated according to the formula:

$$\% \text{ inhibition} = \left[1 - \frac{R_t - R_c}{R_{max} - R_c} \right] \times 100$$

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in which R_T was the average cpm of the experimental well with inhibitors. R_c was the

average background cpm and R_{max} was the average maximal binding without any inhibitors.

         If Ab3 has a similar binding site as Ab1, it should compete with Ab1 for binding to

CEA on LS174-T cells. A fixed amount of radiolabeled Ab1 was co-incubated with different

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amounts of purified Ab3 or control Ab3 preparations and LS174-T cells (Figure 23). One

hundred and fifty nanograms of purified Ab3 inhibited binding by 50% and 1 μg of purified

Ab3 gave over 80% inhibition, whereas the control Ab3 used at a 5 μg concentration did not

produce any inhibition. Similarly, 50% inhibition of binding was obtained with 93 ng of

Ab1. These results indicate that Ab2-immune monkey antibody binds to the same antigen as

Ab1 and therefore the Ab3 preparation contains antibody molecules with Ab1 properties.

<u>Example 4</u>

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<u>Analysis of Immune Response Elicited</u>

<u>in Humans With Advanced CEA-Associated Disease By 3H1</u>

Selection of Patients

Twelve patients participated in the study (Table 2). All of the patients had CEA

positive advanced colorectal carcinoma and failed standard therapies (Table 2).

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Table 2
Patient Characteristics

Patient No.	Age/Sex	Dosage (mg)	No. Doses	Metastatic Disease	Baseline CEA Level	Humoral Response	Cellular	Off Study Study	Why Off
1	72/M	4	7	lung	160	+	+	03/10/94	progression
2	43/F	2	4	liver	110	+	+	12/09/93	progression
3	46/F	1	4	lung, liver	140	+	+	12/02/93	progression
4	61/F	2	4	lung, ileum	60	-	-	12/11/93	progression
5	60/M	1	7	lung, liver	3	+	-	05/02/94	progression
6	68/M	4	8	lung, liver	81	+	-	05/16/94	progression
7	47/M	2	4	liver	15	+	+	03/17/94	progression
8	80/F	4	4	liver	42	-	-	03/17/94	progression
9	51/M	4	4	liver	210	+	+	04/07/94	progression
10	36/M	1	8	pelvis	1	-	-	06/28/94	progression
11	70/M	4	12	lung	58	+	+		progression
12	53/F	2	5	lung, liver	35	+	+	06/09/94	progression

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Baseline studies included complete physical examination, chest radiography, computer axial

tomography examination of the abdomen, serum CEA level, routine blood counts and

chemistries. All of the patients had been off prior therapy for at least four weeks and staging

was repeated at the conclusion of therapy.

5   *Treatment Schedule*

The patients were treated intracutaneously with either 1 mg, 2 mg or 4 mg of

aluminum hydroxide precipitated 3H1 (Example 2) every other week for four injections. If

the patients were stable at the end of the four injections, they were then continued with

injections or a monthly basis and evaluated every three months. Patients were removed from

10   study if they demonstrated growth of their tumor.

Preparation of Ab2

3H1 was obtained as described in Example 1 and alum-precipitated as described in

Example 2. The final product was tested for sterility, pyrogenicity and general safety in

guinea pigs before use. An Investigational New Drug Application was approved through the

15   United States Food and Drug Administration (BB-IND 5055). Before administration, 3H1

was heat treated in the presence of adjuvant at 45°C for 30 minutes in a water bath.

Purified CEA

Purified CEA was obtained commercially from Rougier Bio-tech, Montreal, Canada

(cat. no. 70015). CEA was isolated from human liver metastasis of colonic tumors by

20   perchloric acid extraction and purified twice by ion-exchange chromatography followed by

gel filtration and several steps of HPLC chromatography. The CEA is 100% pure, produced

a single band at 180,000 m.w. by high power liquid chromatography and SDS-PAGE and

was immunoprecipitated as a single band by horse as well as rabbit anti-CEA antibody. The

CEA preparation was resolved into two closely migrating bands at 180,000 and 200,000 m.w.

25   by Western blot analysis using murine monoclonal antibody anti-CEA. We rechecked the

material by Western blot analysis using monoclonal antibody 8019.

Toxicity and Clinical Responses

Toxicity was minimal with only local reactions at the injection site with mild erythma and induration and mild fever and chills relieved by acetaminophen. The anti-idiotypic treatment did not have any deleterious effect on hematopoietic cells, renal or hepatic

5   function. Patients were monitored very closely for disease activity. Eleven patients have had progressive disease (Table 2). The remaining patient was stable at ten months into therapy.

Serial Monitoring of Circulating CEA

Indirect measurement of extent of disease (CEA level) was recorded prior to immunization and determined after each immunization and then once monthly following

10   completion of the immunization schedule. For this, patients' sera were heat-inactivated to precipitate the immunoglobulins which would interfere with the CEA monitoring assays involving murine monoclonal Ab1. CEA is heat stable, and was measured in the clear centrifuged supernatant by routine assay. The serial monitoring of CEA correlated with disease progression and all patients who clinically progressed and a rise in their serum CEA

15   levels except patients five and ten who did not secrete CEA.

For quantification of CEA in heat-extracted serum, 1 ml of 0.2 M sodium acetate buffer, pH 5.0 was added to 0.5 ml of serum, vortex-mixed, incubated for 15 min. at 900C, and centrifuged (1200 X g, 10 min). The supernatants were assayed the same day or stored frozen at - 20°C until assay. One hundred microliters of supernatant was then assayed by the

20   enzyme immunoassay for CEA as described (Hansen et al. (1989) *Clin. Chem*. 35(1):146–151).

Assays for Humoral Immunity

(a)   Total anti-3H1 response

The development of humoral immunity induced by immunization with alum-

25   precipitated 3H1 was assessed by testing sera obtained from patients before therapy and after each treatment with the vaccine. The sera were initially tested for total human anti-murine-antibody responses including anti-iso/allo/and anti-anti-idiotype antibodies by sandwich

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radioimmunoassay as described by Hansen et al. ((1994) Clin. Chem. 35(1):146-151.

Briefly, microtiter plates were coated with 3H1 and incubated with different dilutions of

patients' sera. After washings, the antigen-antibody reaction was tagged using ^{125}I-labeled

anti-Id 3H1 in a homogeneous sandwich radioimmunoassay. Since 3H1 is injected as intact

5   IgG1, patients are expected to mount human anti-mouse antibody responses.

Hyperimmune sera (following the fourth injection of 3H1) from nine of twelve

patients showed significant levels of total human anti-mouse antibody responses including

anti-iso/allo/and anti-anti-idiotypic antibodies against immunizing Ab2, 3H1, as determined

by homogeneous sandwich radioimmunoassay.

10   (b)   Specific Ab3 Response to Ab2

Next the sera from these immunized patients were checked for their ability to inhibit

the binding of ^{125}I-labeled Ab1. 8019, to Ab2 3H1 on the plate by radioimmunoassay or vice

versa (inhibition of radiolabeled Ab2 binding to Ab1 on the plate). These reactions were

done in the presence of excess normal murine immunoglobulin to block human antibodies

15   against isotypic and allotypic determinants.

Crude sera obtained from patients after the fourth treatment were pre-incubated with

normal murine immunoglobulin to block human antibodies against isotypic and allotypic

determinants. We routinely used post fourth immunization because this was the number of

injections all 12 patients received. For patients who received more than four injections,

20   immune responses remained comparable or continued to increase in titer. Serial dilutions of

sera were then tested for inhibition in the Ab1-Ab2 binding assay. All assays were

performed in triplicate. For direct binding inhibition assay between Ab1 and Ab2, purified

3H1 (Ab2) was used to coat plates (500 mg/well) and the binding of radiolabeled 8019 (Ab1)

to Ab2 was tested for inhibition in the presence of different patients' hyperimmune Ab3 sera

25   and Ab1. This demonstrated whether Ab3 in patients' sera shared idiotopes with 8019 (Ab1).

Also, this inhibition assay between Ab1-Ab2 binding by Ab3 sera indicated whether Ab3 is a

true anti-anti-idiotype. Unrelated Ab2 was used as control. After washings, the antigen-

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antibody reaction was tagged using ^{125}I-labeled anti-idiotype reagent in a homogeneous

sandwich radioimmunoassay as above. Pretreatment, nonimmune sera and sera from normal

donors were used as controls.

Figure 23 demonstrates representative data from the first five patients. Sera from

5   patients No. 1, 2, 3 and 5 at 1/10 dilution, inhibited binding of iodinated 8019 to 3H1 by 62 -

100 percent and inhibition of binding decreased with increasing dilution of the sera. Sera

from patient No. 4 showed minimal non-specific inhibition at all dilutions used and pre-

immune sera showed no inhibition. Although steric hindrance by Ab3 binding can not be

excluded in these assays, the results suggest the presence of true anti-anti-idiotypic antibodies

10   that share idiotypes with Ab1. Again, nine out of twelve patients were positive for Ab3

responses by this assay.

(c)   Induction of Anti-CEA Antibodies by 3H1

This assay was conducted to determine whether some of the Ab3 induced in patients

by 3H1 Ab2 were of the Ab1 type and will bind to CEA. A pure preparation of CEA

15   obtained from Rougier Biotech (as described above) was used to reduce the risk of obtaining

false positive results due to nonspecific binding. Purified CEA was radioiodinated with [125]I

by the Chloramine T method. Radiolabeled CEA (1 X 10^6 cpm) was reacted with 0.5 ml of

patient's serum pre-adsorbed on protein G-Sepharose beads. After reactions, the beads were

washed and counted in a gamma-ray spectrophotometer. Each sample was performed in

20   duplicate and the mean of the cpm bound is shown. Pre-immune sera, phosphate buffered

saline-bovine serum albumin, as well as Ab3 sera obtained from a patient treated with an

unrelated murine monoclonal antibody for T cell lymphoma were used as controls in these

assays.

As shown in Figure 25, immunization with 3H1 induced antibodies that bound to

25   radiolabeled CEA. Nine of twelve patients developed anti-CEA antibodies measurable by

this assay. Patients No. 4, 8 and 10 were anergic for human anti-mouse antibody response

and did not produce antibodies against CEA, while patients No. 1, 2, 3, 5 and 12 showed high

binding, and patients No. 6, 7, 9 and 11 showed binding greater than the background count

obtained with PBS-BSA (Sample No. 13) or pre-immune sera (data not shown). Sample

No. 14 was used as a positive anti-CEA (8019) control. Pre-treatment, non-immune sera and

sera from normal donors were used as controls in these assays.

5   (d)   Flow Cytometry (FACS) Analysis with Ab1 and Patient's Ab3

To determine the reactivity with cell-cultured surface CEA, CEA-positive colorectal

cancer derived LS174-T cells (1 X 10^6 per well) and CEA-negative B cell lymphoma, Raji

cells (1 x 10^6 per well) were reacted with Ab1 (8019) and patient's immune sera (Ab3) at

1:100 dilution at 4°C for 60 minutes. After washing, the cells were incubated with either

10   goat anti-human or goat anti-mouse F(ab')₂ IgG-FITC labeled antibody (Tago) for 30 minutes

at 40°C. They were then washed twice, fixed in 2 percent paraformaldehyde and analyzed by

flow cytometry (FACS Star, Becton Dickinson). Preimmune patient's sera were used as a

control.

As shown in Figure 26, crude sera from a representative 3H1-immunized patient

15   bound to LS174–T cells (A) similar to the binding pattern obtained with 8019 (B) and did not

bind to human B cell lymphoma cells which do not express CEA (Fig. 3C). Similar results

were found with all of the positive patients.

(e)   Competition of Ab1 and Patients' Ab3 for Binding to LS 174-T Cells

If Ab3 has a similar binding site as Ab1, it should compete with Ab1 for binding to

20   CEA on LS174-T cells. A fixed amount of radiolabeled 8019 (~90,000 cpm) was co-

incubated with different concentrations of patient's purified Ab3 or Ab1 preparations and

LS174-T cells.

Ab3 was purified from patients' sera as follows. Fifty milliliters of hyperimmune

serum were passed over an immunoadsorbent column consisting of immunizing anti-idiotype

25   immunoglobulin (3H1) coupled to Sepharose 4B. Anti-anti-idiotypic antibodies (Ab3)

bound to the column were eluted with 0.1 M glycine-hydrochloric acid buffer (pH 2.4). The

eluted antibody was neutralized with 3M Tris, dialyzed against PBS, pH 7.2 and then passed

over an immunoadsorbent column consisting of allotype matched normal mouse

immunoglobulin coupled to Sepharose 4B to remove anti-isotypic and anti-allotypic

reactivities. Antibody that passed through was concentrated and used as purified Ab3. The

isotope of Ab3 was determined by ELISA using human anti-isotope specific reagents (Tago).

5   As Figure 27 shows. purified 8019-IgG1 (Ab1) inhibited binding by 80% at 0.75 µg

whereas patient's purified Ab3 (from patient No. 1) produced 60 percent inhibition at the

same concentration. Overall. the inhibition curves obtained with Ab1 and Ab3 were very

similar at different dilutions. This indicated that the patient's Ab3 bound to the same

antigenic epitope as Ab1 and therefore contained antibody molecules with Ab1 properties.

10   (f)   Immunoprecipitation of CEA by Ab1 and Ab3

Purified CEA was labeled with ^{125}I by The Chloramine T-method and reacted with

purified Ab3 (10 µg) or Ab1 (10 µg) or unrelated control Ab3 from lymphoma patient (10 µg)

or PBS-BSA control, previously adsorbed on to protein G-Sepharose beads. After washings,

the antigen-antibody coated beads were analyzed by SDS-PAGE according to the method of

15   Lamella ((1970) Nature 227:680-685) and radioautographed.

It had been previously shown that Ab1 8019 specifically immunoprecipitated the

180,000 m.w. CEA by SDS-PAGE analysis (Bhattacharya-Chatterjee (1990)). To confirm

that the Ab3 induced by 3H1 was specific for the CEA molecule, the iodinated purified CEA

preparation was immunoprecipitated by purified Ab3 preparations obtained from two patients

20   as well as Ab1 and analyzed by SDS-PAGE. The results in Figure 27 indicate that both

patient's Ab3 (Lane 2 and 3) precipitated the same 180,000 m.w. CEA band as that of murine

Ab1 8019 (lane 1). There was no cross-reactivity (lane 4) when the iodinated CEA was

reacted with purified Ab3 obtained from a patient treated with an unrelated Ab2 (4DC6).

When the iodinated CEA antigen, pretreated with either of the two positive patients' Ab3

25   preparations. was reacted with 8019, there was no significant immunoprecipitation

suggesting that the iodinated preparation was depleted of the CEA antigen (data not shown).

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(g)   Immunoreactivity of Ab3 with Tumor Sections

The reactivities of monoclonal Ab1 (8019) and purified Ab3 at 10 μg/ml solution

were compared on autologous and allogenic surgical specimens of colonic adenocarcinomas

from the patients by immunoperoxidase assay. a very sensitive staining method

5   (biotinstreptavidin reagents. Vector. Burlingame. CA) as described in detail elsewhere

(Bhattacharya-Chatterjee (1990)). All sections were counterstained with Meyer's

hematoxylin. Pertinent specificity tests were performed. including block of the endogenous

peroxidase, omission of the first layer. or substitution of nonimmune homologous serum for

the specific antiserum and P3-653 myeloma culture supernatant as the control.

10   The pattern of reactivity of patient Ab3 on autologous malignant colon tissues was

identical to that obtained with allogeneic tumor specimens (Figures 28–1 and 28–2,

respectively). Ab1 8019 showed identical staining patterns (Fig. 28–3), whereas there was no

reactivity with control Ab3 obtained from a patient treated with an unrelated Ab2 (4DC6)

(Fig. 28–4). Reactions with Ab1 or purified Ab3 (Fig. 28–1, 28–2, 28–3) resulted in the

15   staining of both tumor cells as well as secreted mucinous materials. The staining was apical

in gland-like structures and granular (cytoplasmic) in less differentiated areas. There was no

reactivity of Ab1 and purified Ab3 on normal tissues from colon (Figure 28–5 and 28–6),

cecum, duodenum, stomach striated muscle or smooth muscle.

Assay for T cell Proliferative Response

20   Cellular immune responses were measured by the proliferation of peripheral blood

mononuclear cells incubated with aluminum hydroxide precipitated anti-idiotype antibody

3H1 and aluminum hydroxide precipitated isotope matched control anti-idiotype antibody

4DC6.

Peripheral blood mononuclear cells were isolated from blood obtained after four

25   immunizations by standard Ficoll-Hypaque density gradient centrifugation method and 5 X

10^6 cells per well were incubated with different concentrations of GH1-Alugel and control

4DC6-Alugel (10μg to 2μg) in RPMI medium with 5 percent heat-inactivated fetal calf serum

and penicillin and streptomycin. The non-specific mitogen phytohemagglutinin-P was used as a positive control at 2μg and 1μg per well. After the cells were incubated for five days at 37°C in an atmosphere containing 5 percent carbon dioxide, they were pulsed with ^{3}H-thymidine (1 μCi per well) for 20 hours. 3H-thymidine incorporation was measured in pre

5   and post-therapy samples. Data were expressed as mean counts (triplicate wells) per minute of ^{3}H-thymidine incorporation. The Standard Deviation of the data was <10% for each determination.

Peripheral blood mononuclear cells isolated from some selected patients were also incubated with different concentrations of purified CEA (10 ng to 250 ng) as per protocol

10   above.

Positive proliferative responses were seen in seven of twelve patients. All seven of these patients developed an Ab3 antibody response (Table 2). Representative data from two patients (No. 1 and 12) are shown in Figure 30. Pre-immune cells had no proliferative response to the anti-idiotype antibody while hyperimmune cells had a significant response.

15   Four of the seven responding patients (two treated with a 2 mg dose and two with a 4 mg dose) also showed T cell proliferation in the presence of purified CEA suggesting antigen specific T cell response. There was also a response to the isotope matched 4DC6 aluminum hydroxide-precipitated anti-idiotype antibody; this response was significantly less than that of the 3H1 response, likely representing a response to the non-idiotype components of the

20   immunoglobulin molecule. The difference in the response to 3H1-Alugel compared to control 4DC6-Alugel was significant (p<0.003) as was the response to CEA compared to BSA (p<0.005). There was no response to alugel itself (data not shown). Flow cytometric analysis of the cultures demonstrated that greater than 90% of the proliferating cells were CD4 positive T lymphocytes. The three patients who were anergic for human anti-mouse

25   antibody response also did not demonstrate any T cell proliferative response. Of the five non-responders, three were treated with 1 mg, one with 2 mg and one with 4 mg dosage of 3H1-Alugel.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the

5   description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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WHAT IS CLAIMED IS:

1. A monoclonal anti-idiotype antibody 3H1 produced by a hybridoma cell line ATCC No. HB12003 and progeny thereof.

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2. The antibody of claim 1, further comprising a label capable of producing a detectable signal.

3. A hybridoma cell line designated ATCC No. HB12003 and progeny thereof.

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4. A hybridoma cell line that produces a monoclonal anti-idiotype antibody having a light chain variable region amino acid sequence identical to SEQ ID NO:2 and a heavy chain variable region amino acid sequence identical to SEQ ID NO:4.

15   5. A monoclonal anti-idiotype antibody having a light chain variable region amino acid sequence identical to SEQ ID NO:2 and a heavy chain variable region amino acid sequence identical to SEQ ID NO:4.

6. A monoclonal anti-idiotype antibody having a light chain variable region encoded

20 by a polynucleotide encoding an amino acid sequence identical to SEQ. ID. NO:2 and a heavy chain variable region encoded by a polynucleotide encoding an amino acid sequence identical to SEQ. ID NO:4.

7. A pharmaceutical composition comprising an effective amount of monoclonal

25 anti-idiotype antibody 3H1 in a pharmaceutically acceptable excipient.

8. A vaccine comprising an effective amount of monoclonal anti-idiotype antibody 3H1.

9. The vaccine of claim 8. further comprising an adjuvant.

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10. The vaccine of claim 9. wherein the adjuvant is aluminum hydroxide.

11. A method of eliciting an immune response in an individual with advanced carcinoembryonic antigen (CEA) associated disease comprising the step of administering an

10 effective amount of monoclonal anti-idiotype antibody 3H1 to the individual.

12. The method of claim 11. wherein the immune response is the production of anti-CEA antibody.

15   13. A method of eliciting an immune response in an individual with advanced CEA-associated disease comprising the step of administering the vaccine of claim 9 to the individual.

14. The method of claim 13. wherein the immune response is the production of anti-

20 carcinoembryonic antigen.

15. A method for detecting the presence of an anti-CEA antibody bound to a tumor cell comprising the steps of contacting the tumor cell with monoclonal antibody according to claim 1 for a sufficient time to allow binding to the anti-CEA antibody, and detecting the

25 presence of any 3H1 which is bound to the anti-CEA antibody.

16. A method for detecting the presence of an anti-CEA antibody bound to a tumor
cell comprising the steps of contacting the tumor cell with the monoclonal anti-idiotype
antibody according to claim 5 for a sufficient time to allow binding to the anti-CEA antibody,
and detecting the presence of any 3H1 which is bound to the anti-CEA antibody.

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17. A diagnostic kit for detection or quantitation of an anti-carcinoembryonic antigen
antibody comprising a monoclonal anti-idiotype antibody according to claim 1 in suitable
packaging.

10   18. A diagnostic kit for detection or quantitation of an anti-carcinoembryonic antigen
antibody comprising a monoclonal anti-idiotype antibody according to claim 5 in suitable
packaging.

19. A method for detecting an anti-carcinoembryonic antigen response in an
15 individual comprising contacting a biological sample from the individual with the
monoclonal antibody of claim 1.

20. A method for detecting an anti-carcinoembryonic antigen response in an
individual comprising contacting a biological sample from the individual with the
20 monoclonal anti-idiotype antibody of claim 5.

21. A method of palliating carcinoembryonic antigen-associated disease in an
individual having advanced carcinoembryonic antigen-associated disease comprising
administering an effective amount of monoclonal antibody 3H1 to the individual.

25

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3H1L.SEQ

TCA TAT GGA TTA CTA GTC GAC

ATG GTA TCC ACA GCT CAG TTC CTT GGT ATC TTG TTG CTC TGG TTT CCA GGT

ATC AAA TCT GAC ATC AAG ATG ACC CAG TCT CCA TCT TCC ATG TAT GCA TCT

CTA GGA GAG AGA GTC ACG ATC ACT TGC AAG GCG AGT CAG GAC ATT AAT GGT

TAT TTA AAT TGG TTC CAA CAA GAA CCA GGG AAA TCT CCT AAG ACC CTG ATC

TAT CGT GCA AAT AGA TTG ATA GAT GGG GTC CCA TCA AGG TTC AGT GGC AGT

GGA TCT GGG CAA GTT TAC TCT CTC ACC ATC AGC AGC CTG GAA TAT GAA GAT

ATG GGA ACT TAT TAT TGT CTA CAG TTT GAT GAG TTT CCG TGG ATG TTC GGT

GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT GAT GCT GCA CCA ACT GTC TCC

ATC TTC CCA CCA TCC AGT

Fig. 1A

3H1L.pep
MVSTAQFLGILLLWFPGIKS
DIKMTQSPSSMYASLGERVTITC
KASQDINGYLN
WFQQEPGKSPKTLIY
RANRLID
GVPSRFSGSGSGQVYSLTISSLEYEDMGTYYC
LQFDEFPWMFGGGTKLEIK
RADAAPTVSIFPPSS

Fig. 1B

3H1H.SEQ

AGTCATATGGATTGGGAATTC

ATG GAA TGG AGC TGG GTC ATT CTC TTC CTC CTG TCA GGA ACT GCA GGT

GTC CAC TCT GAG GTC CAG CTG CAA CAG TCT GGA CCT GAG CTG GTG AAG CCT

GGA GCT TCA CTG AAG ATT TCC TGC GAG GCT TCT GGT TAC TCA CTC ACT GCC

TAC ACC ATG AAC TGG GTG AAG CAG AGC CAT GGA AAG AGC CTT GAG TGG GTT

GGG CTG ATT AAT CCT TTC AGT GGT GAT ACT AAC TAC AGC CAG AAA TTC ACG

GGC AAG GCC ACA TTA ACT GTA GAC AGG TCA TCC AGC ACA GCC TAC ATG GAG

CTC CTC AGT CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GTC ATT ACT

CCG GTT CCC TAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC

GTC TCC TCA GCC AAA ACG ACA CCC CCA TCC GTC TAT

Fig. 2A

3H1H.

MEWSWVILFLLSGTAGVHS

EVQLQQSGPELVKPGASLKISCEASGYSLT

AYTMN

WVKQSHGKSLEWVG

LINPFSGDTNYSQKFTG

KATLTVDRSSSTAYMELLSLTSEDSAVYYCVI

TPVPYWYFDV

WGAGTTVTVSS

AKTTPPSVY

Fig. 2B

```
                                                                      -100      -90
                                                                   TGTGTGTGTG  TATATAACAT  BALB/c

                                 MVCP-2->
  -80         -70        -60        -50        -40        -30        -20        -10
AACTGTTTAC A..CATAATA CACTGAAATG GAGCCCTTCC CTGTTACTTC ATACCATCCT CTGTGCTTCC TTCCTCAGGG  BALB/c
---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  CE
---------- -.-------- ---------- ---------- ---------- ---------- ---------- ----------  CBA
-- -------- ---------- ---------- --------T- ---------- ---------- ---------- ----------  RIII
---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  DBA/2
------ --T------- ---------- xx------T- ---------- ---------- ---------- ----------  C57BL
---- ---------- ---------- ---------- ---------- ---------- ---------- ----------  A/J
-- ---------- --T------- -x------xxT ---------- ---------- ---------- ----------  NZB
                                                          ----- ---------- ----------  C58
-- ---------- ---------- ---------- ---------- ---------- ---------- ----------  AKR
                                                                                        PL
---------- ---------- ---------- ---------- ---------- ---------- ----------  SJL
---------- -.-------- ---------- ---------- ---------- ---------- ---------- ----------  M.spretus
GT--A-A.-- -TG----G ----------..... .....TT---- ---------- ---------- ---------- ----------  LOU
GT--A-A.-- -TG----G -----------..... .....TT---- ---------- ---------- ---C------- ----------  DA

 109  110                        115                      120                      125
GCT GAT GCT GCA CCA ACT GTA TCC ATC TTC CCA CCA TCC AGT GAG CAG TTA ACA TCT GGA  BALB/c
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CE
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CBA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  RIII
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  DBA/2
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C57BL
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  A/J
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  NZB
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C58
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  AKR
-T- --- x-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  PL
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  SJL
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  M.spretus
--- --- --- --- --- --- --T --- --- --- --- --- -CG --A --- --- G-- A-- ---  LOU
--- --- --- --- --- --- --- --- --- --- --- --- -TG --A --- --- --- --- ---  DA

                                                                          MVCP-45
   130                       135                      140                      145
GGT GCC TCA GTC GTG TCC TTC TTG AAC AAC TTC TAC CCC AAA GAC ATC AAT GTC AAG TGG  BALB/c
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CE
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CBA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  RIII
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  DBA/2
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C57BL
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  A/J
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  NZB
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C58
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  AKR
--- --- --- --- --- --- --- --- --- --- --- --- -x-- --- --- --- --- --- ---  PL
--- --- --- --- --- --- --- --- --- --- --- --- -G-- --- --- --- --- --- ---  SJL
--- --- ---. --- --- --- --- --- --- --- --- --- -G-- --- --- --- --- --- ---  M.spretus
--- --- --- --- --- --- C-- A-- --- --- --- --T --- -G- --- --- -G- --- --- ---  LOU
--- --- A-- --- --- --- G-- --- --- --- --T --- -G- --- --- -G- --- --- ---  DA

          MVCP-45->
   150                       155                      160                    165
AAG ATT GAT GGC AGT GAA CGA CAA AAT GGC GTC CTG AAC AGT TGG ACT GAT CAG GAC AGC  BALB/c
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CE
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CBA MK
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  RIII
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  DBA/2
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C57BL
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  A/J
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  NZB
--- --- --- --- --- --- --- --- --T T-- xxx --- --- --- --- --- --- --- ---  C58
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  AKR
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  PL
--- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- ---  SJL
--- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- ---  M.spretus
--- --- --- --- -C- --- --- -G- G-- --T --- --- G-- --- GTT --- --- --- --- ---  LOU
--- --- --- --- --- -A- -G- G-- --T --- --- G-- --- GTT --- --- --- --- ---  DA
```

Fig. 3A

```
      170                    175                    180                    185
AAA  GAC AGC ACC TAC AGC ATG AGC AGC ACC CTC ACG TTG ACC AAG GAC GAG TAT GAA CGA  BALB/c
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CE
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CBA
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  RIII
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  DBA/2
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C57BL
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  A/J
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  NZB
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C58
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  AKR
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  PL
---  --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- ---  SJL
---  --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- ---  M.spretus
---  --- --- --G --- --- --- --- --- --- T-- --- --- --- -CT --C --- --- A-T  LOU
---  --- --- --G --- --- --- --- --- --- T-- --- --- --- -TT --A --- --- A-G  DA

      190                    195                    200                    205
CAT  AAC AGC TAT ACC TGT GAG GCC ACT CAC AAG ACA TCA ACT TCA CCC ATT GTC AAG AGC  BALB/c
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CE
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  CBA
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  RIII
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  DBA/2
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  C57BL
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  A/J
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  NZB
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- ---  C58
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- ---  AKR
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- ---  PL
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- ---  SJL
---  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  M.spretus
---  --- CT- --- --- --- --- -TT GT- --T --- --- --- T-C --- --- G-C --- --- ---  LOU
---  --- CT- --- --- --- --- -TT GT- --T --- --- --- T-C --- --- G-C --- --- ---  DA
                                                              <-MXC-C
```

```
                                   <-MXC-B
                                 <-MXC-A
      210              214       +10        +20        +30        +40        +50        +57
TTC  AAC AGG AAT GAG TGT TAGAGACAAA GGTCCTGAGA CGCCACCACC AGCTCCCCAG CTCCATCCTA TCTTCC  BALB/c
---  --- --- --- --- --- ----------  --                                                CE
---  --- --- --- --T --- ----------                                                    CBA
---  --- --- --- --- --- ----------                                                    RIII
---  --- --- --- --- --- ----------                                                    DBA/2
---  --- --- --- --- --- ---------- --                                                 C57BL
---  --- --- --- --- --- ----------                                                    A/J
---  --- --- --- --- --- ---------- ---                                                NZB
---  --- --- --- -T- -x- ----------                                                    C58
---  --- --- --- --- --- ----------                                                    AKR
---  --- --- --- --- --- ----------                                                    PL
---  --- --- --- --- --- ----C----                                                     SJL
---  --- --- --- --- --- ----C---- ---------- ---------- ---------- ---------- ------  M.spretus
---  --- --- --- --- --- ----CC---- ----------G T------T.. .---------- ----T---A- ------  LOU
---  --- --- --- --- --- ----CC---- ----------G T------T.. .---------- T---T---A- ------  CA
```

Fig. 3B

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```
                         20              40              60              80                    A
  0  TCGGGGACATGGGAAGGGTGCAAAAGTAGCGGCCTTCTAGAAGGTTTGGACCTGTCCTGTCCTGTCCGACAGTGTAATCACATATACTTTTTCTTGTAGC
                                     T                          N     A  S  Q
          K  T  T  P  P  S  V  Y  P  L  A  P  S  S  A  A  Q  T  N  S  M  V  T  L  G  C  L  V  K  G  Y  F  P
100  CAAAACGACACCCCCATCTGTCTATCCACTGGCCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTCAAGGGCTATTTCCCT

          E  P  V  T  V  T  W  N  S  G  S  L  S  S  G  V  H  T  F  P  A  V  L  Q  S  D  L  Y  T  L  S  S  S
200  GAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAG

             S     T     -  -  -                          A  P
          V  T  V  P  S  S  P  R  P  S  E  T  V  T  C  V  V  A  N  P  A  S  S  T  K  V  D  K  K  I
300  TGACTGTCCCCTCCAGCCCTCGGCCCAGCGAGACCGTCACCTGCAACGTTGCCCACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGGTGAGAGGAC

400  ATATAGGGAGGAGGGGGTTCACTAGAAGTGAGGCTCAAGCCATTAGCCTGCCTAAAACCAACCAGGCTGGACAGCCAACCAACCAGGAAATGGATCTCAGCC

500  CAGAAGATCAAAAGTTGTTCTTCTCCCTTCTGGAGATTTCTATGTCCTTTACAACTCAATTGGTTAATATCCTGGGTTGGAGTCCCACACATCTTGACAA

600  ACAGAGACAAATTTGAGTATCACCAGCCAAAAGTCATACCCAAAAACAGCCTGGCATGACCACACACCAGACTCAAACTTACCCTACCTTTATCCTGGTG

                                                V  F  R  D  C  G  C  K  P  C  I  C  T
700  GCTTCTCATCTCCAGACCCCAGTAACACATAGCTTTCTCTCCACAGTGCCCAGGGATTGTGGTTGTAAGCCTTGCATATGTACAGGTAAGTCAGTGGCCT

                                                                               V  P  E  V  S  S
800  TCACCTGACCCAGATGCCAACAAGTGGCAATGTTGGAGGGTGGCCAGGTATTGACCTATTTCCACCTTTCTTCTTCATCCTTAGTCCCAGAAGTATCATCT

                          T     L        V
          V  F  I  F  P  P  K  P  K  D  V  L  T  I  T  L  T  P  K  V  T  C  V  V  V  D  I  S  K  D  D  P  E
900  GTCTTCATCTTCCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGACATCAGCAAGGATGATCCCGAGG

                    N                                                        V        A
          V  Q  F  S  W  F  V  D  D  V  E  V  H  T  A  Q  T  Q  P  R  E  E  Q  F  N  S  T  F  R  S  V  S  E  L
1000  TCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGTGCACACAGCTCAGACGCCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGCTCAGTCAGTGAACT

          P  I  M  H  Q  D  W  L  N  G  K  E  F  K  C  R  V  N  S  A  A  F  P  A  P  I  E  K  T  I  S  K  T
1100  TCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACC

          K
1200  AAAGGTGAGAGCTGCAGTGTGTGACATAGAAGCTGCAATAGTCAGTCCATAGACAGAGCTTGGCATAACAGACCCCTGCCCTGTTCGTGACCTCTGTGCT

                    K     R
          G  R  P  K  A  P  Q  V  Y  T  I  P  P  P  K  E  Q  M  A  K  D  K  V  S  L
1300  GACCAATCTCTTTACCCACCCACAGGCACACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGATGGCCAAGGATAAAGTCAGTCTGA

                                        S  D        A  P                          D
          T  C  M  I  T  D  F  F  P  E  D  I  T  V  E  W  Q  W  S  G  Q  P  A  E  N  Y  E  N  T  Q  P  I  M  M
1400  CCTGCATGATAACAGACTTCTTCCCTGAAGACATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAGCCCATCATGAA

             D
          T  N  G  S  Y  F  V  Y  S  K  L  N  V  Q  K  S  N  W  E  A  G  N  T  F  T  C  S  V  L  H  E  G  L
1500  CACGAATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTG

          H  N  H  H  T  E  K  S  L  S  H  S  P  G  K  term
1600  CACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAATGATCCCAGTGTCCTTGGAGCCCTCTGGTCCTACAGGACTCTGACACCTACCTCC

                    poly A
1700  ACCCCTCCCTGTATAAATAAAGCACCCAGCACTGCCTTGGGACCCTGCAATAACGTCCTGGTGATTTCTGAGATGTAGAGTCTAGCTAGGTCATGGAATG
```
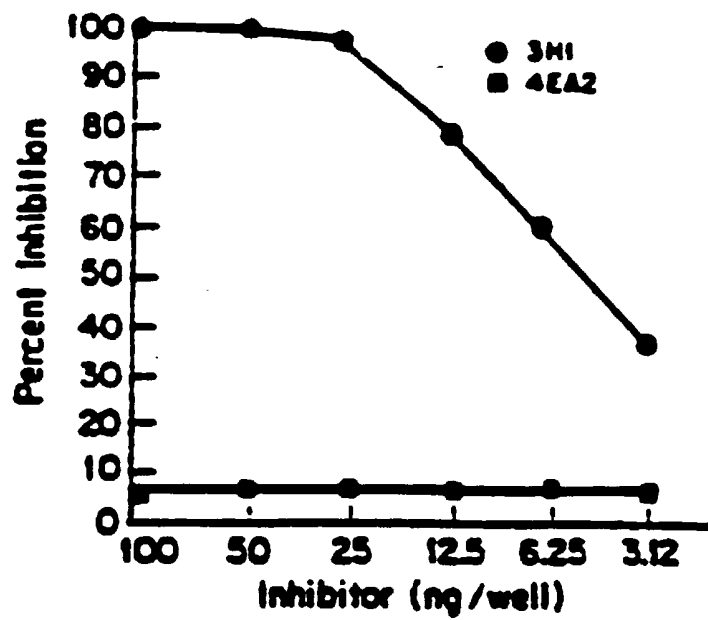
Fig. 4

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Fig. 5

Fig. 6

Fig. 7